

# Illustrations for Health Assessment Techniques of the Atlantic Horseshoe Crab, *Limulus polyphemus*

by  
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## ABSTRACT

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The Atlantic horseshoe crab, *Limulus polyphemus*, is a “living fossil” extant for over 340 million years (Shuster et al. 2003) and is very important for conservation and medical research. The dense egg production during spring spawning along the Northeast coast of the United States coincides with the migratory pattern of a number of species of shorebirds (Shuster et al. 2003) providing an essential source of nutrition for threatened species (Botton 2009; Smith 2012). Cells within the hemolymph, or blood, of horseshoe crabs are harvested for biomedical purposes for the production of Limulus Amebocyte Lysate (LAL), a worldwide standard test for detecting minute amounts of bacterial endotoxins in “biologicals, pharmaceutical drugs, and medical devices” (Smith 2012). No synthetic alternative to LAL exists (Novitsky 2009; Anderson et al. 2013).

Extensive research has been conducted on horseshoe crabs; however, these studies lack guidelines for health examinations, hemolymph evaluations, and necropsy techniques. Unfortunately, the increasing demands of using horseshoe crabs for fishing bait and for hemolymph extraction for LAL production is leading to population decline. Veterinarians and researchers need to know how to properly examine the health of the horseshoe crab and monitor health to keep the species thriving. This study fills that gap through literature research and performed necropsies, of *Limulus polyphemus*.

The results of this study include multiple detailed illustrations explaining internal and external anatomy, common disease states, anatomical sex comparison, inhabiting organisms, hemolymph extraction techniques, book gill anatomy, circulatory system diagram and a necropsy technique guide. This guide explains in detail how to perform a necropsy of the horseshoe crab and the associated anatomy seen during the dissection. It also explains which necropsy approach is best under specific circumstances and what steps to take to preserve important anatomical structures. There has never been a standardized guideline for necropsy techniques, and this series describes the procedure in a comprehensive, anatomically faithful, and sequential manner. These illustrations will

help create a better overall understanding of horseshoe crab anatomy and health leading to proper diagnosis of a disease, thus contributing to proper health maintenance and better conservation strategies.

~Katie Bergdale

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## INTRODUCTION

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Horseshoe crabs are ecologically important for a variety of reasons. Horseshoe crab spawning season along the Northeast coast coincides with the seasonal migration of multiple shorebird species, providing hundreds of thousands of shorebirds with essential nutrients from horseshoe crabs eggs to complete the thousand-mile journey from South America to their arctic breeding grounds (Hurton, et. al., 2009). “The inability of red knots to find adequate food throughout the bay may be having serious effects on their survival” (Botton 2009). Many fish and crustacean species rely on horseshoe crab eggs and trilobite larvae (commonly known as hatchling) as an essential part of their diet (Shuster, et. al., 2003). A variety of species consume juvenile or molting horseshoe crabs, including fish, crustaceans, birds, and marine reptiles (Shuster, et. al., 2003; Botton 2009). Specifically, loggerhead turtles feed extensively on adult horseshoe crabs (Shuster et al. 2003; Seney and Musick, 2007).

Horseshoe crabs are used in fertilizer and livestock feeds and as bait in many whelk and eel fishery companies (Hurton, et. al., 2009; Botton 2009). They have also been widely studied as a model organism for anatomy, physiology, cellular phagocytosis, and the function of its eyes, nervous system, and molting and regeneration abilities (Smith and Berkson, 2005).

Horseshoe crabs play an important role in human health and medicine. Their blue blood, or hemolymph, is harvested and the blood cells, or amebocytes, are extracted and processed to create Limulus Amebocyte Lysate (LAL). This substance is used to test minute amounts of bacteria on medical devices, implants, and vaccines to ensure sterilization (Novitsky 2009). LAL is the most effective way to detect endotoxin, testing at femtogram levels (Hurton, et. al., 2009). In other words, it could find a grain of sand in a football field.

To survive for millions of years, the horseshoe crab has evolved a highly efficient defense system to fight off internal pathogenic parasites (Shuster, et. al., 2003). *Limulus*



blood cells clot the plasma when exposed to gram-negative bacteria. “The majority of the effectors of the immune system are found in the blood or hemolymph, presumably because the blood has ready access to all parts of the body and is best prepared to concentrate defense effectors at a site of pathogenic invasion” (Shuster, et. al., 2003). Another defensive adaptation is the unique location of the nervous system within the arterial system, submerging the nerves within the protective hemolymph. Compared to the highly developed immune defense system of a vertebrate, which contains diverse antibody proteins, *Limulus* depends on an innate immune system containing a single cell, the granular amebocyte (Shuster, et. al., 2003). In the absence of pathogens, cells remain in an inactivated state. In the presence of pathogens, cells release secretory granules that hunt and kill bacteria. Activated cells become motile and attach to other blood cells and surfaces finding and destroying the invading pathogens. This process leads to coagulation of the hemolymph.

W. H. Howell first documented the coagulation of horseshoe crab hemolymph at Johns Hopkins University in 1885 (Shuster, et. al., 2003). Research of clotting mechanisms and immunology continued through the work of Frederik Bang and Jack Levin at the Marine Biological Laboratory (MBL) in Woods Hole in early 1900s (Shuster, et. al., 2003). Their research led to “medical and commercial applications of *Limulus* amebocyte lysate” (Shuster, et. al., 2003).

LAL is a clotting agent created from the separation of inactivated but functioning amebocytes from cellular debris of hemolymph, allowing for accurate testing of sterilization in medical products (Shuster, et. al., 2003). If hemolymph were extracted directly from a horseshoe crab and used for testing, it would immediately clot when exposed to air or any unsterile material due to its high sensitivity for bacteria, providing inaccurate results in sterilization testing. This led to the creation of LAL to prevent unwanted early clotting.

Since the creation of LAL, overharvesting has contributed to the decline in the horseshoe crab population (Hurton, et. al., 2009). The commercial harvest of horseshoe crab hemolymph causes a significant degree of stress to the animal through the biomedical bleeding process. This practice consists of puncturing the heart at the arthroal membrane and draining up to 62% of the blood (Hurton, et. al., 2009). This procedure also leads to other potential stresses such as handling, air exposure, increased temperature, and trauma, causing mortality rates up to 30% (Hurton, et. al., 2009; Anderson, et. al., 2013). There have been no studies to observe the additional consequences and deaths that occur from this invasive process after the horseshoe crabs are returned to the ocean. These population declines cause a significant need to understand the health of the horseshoe crab in order to protect the overall conservation of the species.

The National Aquarium in Baltimore, MD manages several groups of horseshoe crabs, as they are a common species in aquarium collections, representing an important and unusual part of the aquatic ecosystem. The National Aquarium has developed guidelines for health examinations, hemolymph evaluations, and necropsy techniques to better treat and understand horseshoe crabs.

The horseshoe crab has been studied for centuries and is the most researched marine arthropod (Shuster, et. al., 2003). Existing literature, such as *The American Horseshoe Crab* by Carl Shuster, Robert Barlow, and Jane Brockman, *The circulatory system and blood of the horseshoe crab* by Carl Shuster, and *Biology of Horseshoe Crabs* by Kōichi Sekiguchi, provide information on anatomy, physiology, and common diseases. However, these texts do not focus on health exams or hemolymph evaluations. Additionally, many included illustrations are outdated or difficult to interpret. Comprehensive illustrations were found among historical literature including *Recherches sur l'anatomie des Limules* (in french) by Milne-Edwards, 1873, and *Studies on Limulus* by Patten and Redenbaugh,

1899. Both were published in the late 1800s and do not include information on necropsies. These and other existing illustrations of horseshoe crab anatomy are not realistic to color or texture, making it difficult to visualize observations when performing a necropsy.

There is no existing material including standardized guidelines or illustrations depicting necropsy methods using a ventral approach. Unpublished dissection instructions provided by Roxanna Smolowitz, DVM (Roger Williams University) briefly discuss necropsy methods using a dorsal approach, beginning the dissection on the dorsal carapace. This dorsal approach proves difficult due to the extremely dense dorsal carapace, even when using heavy-duty scissors. The ventral approach examined and described in this project proves far easier, as there is a soft spot on the ventral carapace, anterior to the prosomal appendages. The illustrations associated with Dr. Smolowitz's instructions are confusing, poor quality, and difficult to decipher. In practice, it is challenging to preserve important anatomical structures when performing a necropsy on a horseshoe crab without proper guidelines. Tissues are very fragile, making it easy to dissect through important structures. Also, all structures are monochromatic making the anatomy difficult to distinguish. In conclusion, current work on the horseshoe crabs lacks information needed to perform health exams, hemolymph evaluations, and necropsy techniques.

The purpose of this thesis is to provide accurate illustrations to educate veterinarians, aquarists, technicians, and biologists about proper health assessments on horseshoe crabs. All illustrations created correspond with, and directly relate to, the National Aquarium's health assessment guidelines. Understanding both the normal biology and the diseased states of the horseshoe crab will improve development of animal health maintenance and conservation strategies. Information accompanied by instructive illustrations enhances the learning process (Carney, et. al., 2002). Therefore, the current animal care practices developed by the National Aquarium will improve with the addition

of corresponding didactic illustrations. This study includes multiple detailed illustrations of the following subject matter:

- Internal and external anatomy
- Necropsy series and associated anatomy
- Hemolymph extraction and sampling techniques
- Circulatory system
- Proper physical examination for common disease states
- Organisms in/on a horseshoe crab
- Sex comparison
- Cross section of anatomy
- Book gill anatomy
- Leg external anatomy

The primary audience of these illustrations is veterinarians, aquarists, technicians, and biologists who work with horseshoe crabs or have an interest in aquatic animal medicine. The content is presented at a graduate level. The secondary audience is the lay viewer interested in learning more about the anatomy and conservation of horseshoe crabs.

Techniques used in this study included gross dissection to understand anatomy, observation of living specimens to understand common diseases, lesions, symbiotic organisms and animal behavior, and health assessment procedures created and performed by the National Aquarium animal health staff. Information for this thesis was obtained through trips to the National Aquarium in Baltimore, Maryland and a one-week work study at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts.

Nomenclature used in the illustrations is based on the lecture notes *Anatomy and Diseases of Limulus Dissection Instructions*, provided by Dr. Roxanna Smolowitz, Assistant Professor of Biology, and Director of the Aquatic Diagnostic Laboratory at Roger Williams University.

## External Anatomy

While the anatomy of the horseshoe crab has been previously described by numerous authors, this study directly references the lecture notes for the AQUAVET® program at Cornell University, entitled *Anatomy and Diseases of Limulus Dissection Instructions*, by Dr. Roxanna Smolowitz. Additional sources used are identified.

### *Dorsal View*

The horseshoe crab has a protective continuous cuticular body cover, or carapace that is separated into three main sections: prosoma (cephalothorax), opisthosoma (abdomen), and telson (tail). The body is bilaterally symmetrical with a semicircular prosoma. Dorsally, there are two laterally located compound eyes and two medially-anteriorly located ocelli or median eyes. The carapace has a midline keel that begins at the prosoma and continues to the opisthosoma and telson. It is interrupted by the hinge joint that connects the prosoma to the opisthosoma. Laterally and parallel to the midline keel are two longitudinal furrows which start on the prosoma and continue on the opisthosoma, interrupted by the hinge joint. Within each longitudinal furrow on the opisthosoma are six entapophyseal pits that function as attachment points for the genital operculum and gill opercula. Centrally located on the hinge joint is the arthrodiol membrane that is commonly used as the phlebotomy site for hemolymph withdrawal. Medially to both lateral compound eyes is the ophthalmic ridge. The outermost edge of the carapace, the flange, continues around the entire prosoma. The triangular edge of the prosoma is known as the genal angle. The opisthosoma has a movable spine that houses six individual spines on either side. These spines are not controlled voluntarily but are flexible at their attachment points and aid in protection to the opisthosoma. The space between the telson and the posterior triangular tip of the opisthosoma is known as the terminal bay or the exhalant channel. The space between the genal angle and the opisthosoma is known as the inhalant channel. These channels aid in respiration (Fig. 1).

*Ventral View*

On the ventral side, the section of prosoma most anteriorly located is a flat, triangular region called the subfrontal area. The outer ridge of the prosoma that lies anterior to the subfrontal area is known as the doublure. As this ridge extends laterally, it forms an indent known as the exuviation suture.

The horseshoe crab has fourteen pairs of specialized appendages. The anterior six appendages are segmented legs which reside in the vault of the prosoma, while the posterior eight specialized appendages are opisthosomal appendages, including the genital operculum, gill opercula, and telson. The first paired appendages are the chelicerae, which consist of small pinchers specialized for guiding food into the mouth. Anterior to the chelicerae is the ventral sense organ. The second paired appendages are the pedipalps whose anatomy is specific to each sex. Males have larger bulbous claws, while a female's pedipalps are the same as the ambulatory or walking legs. The walking legs comprise the third through fifth appendages and are used in locomotion. The coxa leg segments of the second through the fifth prosomal appendages have regions that together create the gnathobase used for grinding and passing food to the centrally located mouth. The sixth paired appendages are the swimmer legs which are used to propel water to swim forward. Between the pair of swimmer legs are the chilariae.

The flabellum, also called epipodite, articulates with the coxa leg segment of the swimmer leg. The function of the flabellum is still unclear. It is thought to aid in determining low oxygen conditions by testing water composition passing into the book gills through the inhalant water channel (Ecological Research & Development Group, 2002; Fox, 2007; Shuster, et. al., 2003).

The first specialized opisthosomal appendage is the genital operculum, which covers and protects the book gills. The eighth through thirteenth appendages are gill opercula which protect and contain ventrolateral paired book gills, where each contains

hundreds of gill lamellae, or leaflets. Each set of gill opercula have two endopodites which are centrally located and contain branchial warts. The book gills are used for respiration, osmoregulation, and propulsion during swimming. The smooth, slightly raised area on the opisthosoma around the gill opercula is known as the posterior slope and contains the transverse ridge. Lastly, the fourteenth appendage is the telson, which is used to aid the horseshoe crab when flipped upside down and to ward off predators. The anus lies anterior to the telson and is protected by a small ridge known as the axial area (Fig. 2).

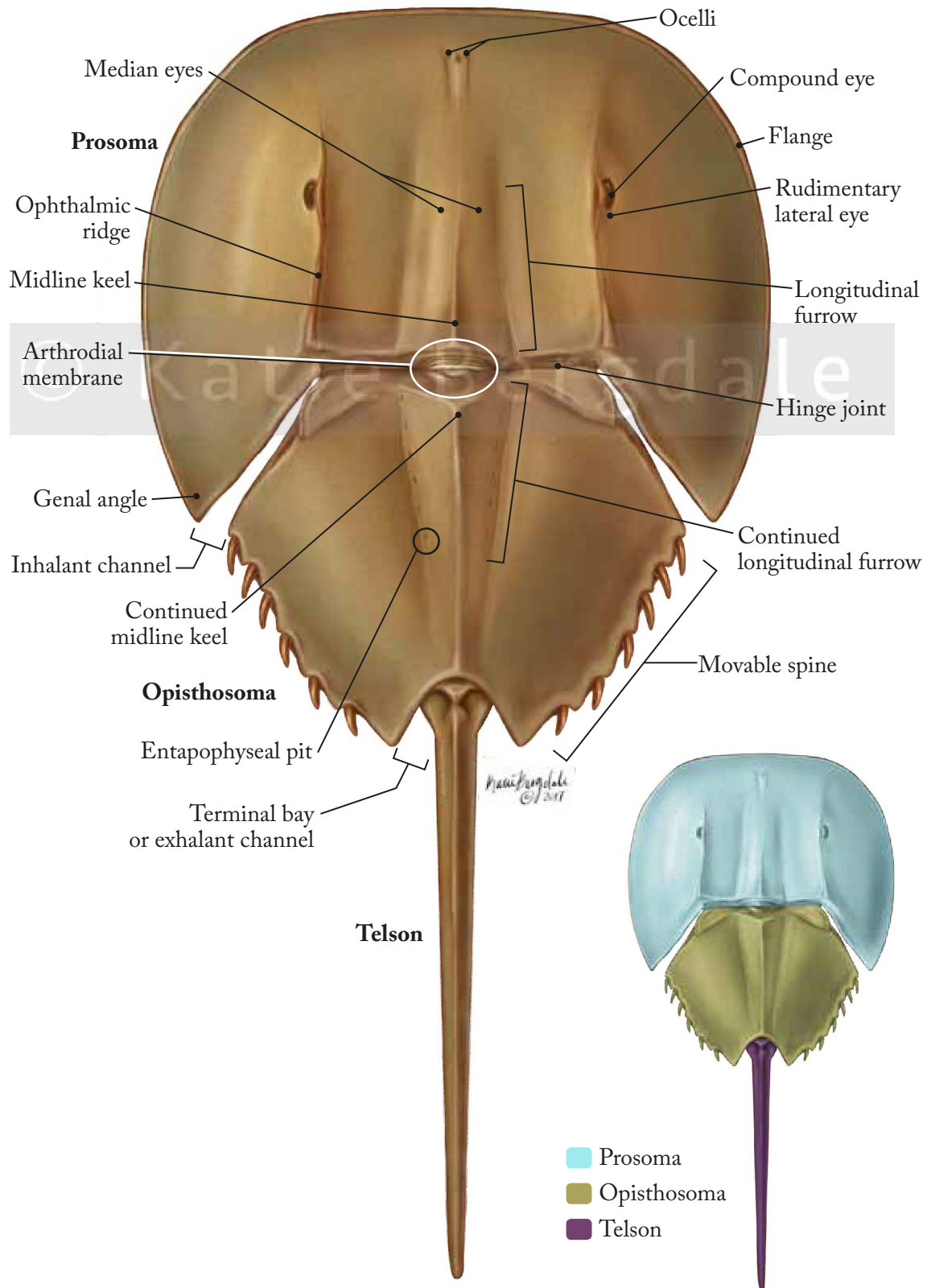


FIG. 1A: External Anatomy: Dorsal view  
FIG. 1B: Segments of the Body: Dorsal view



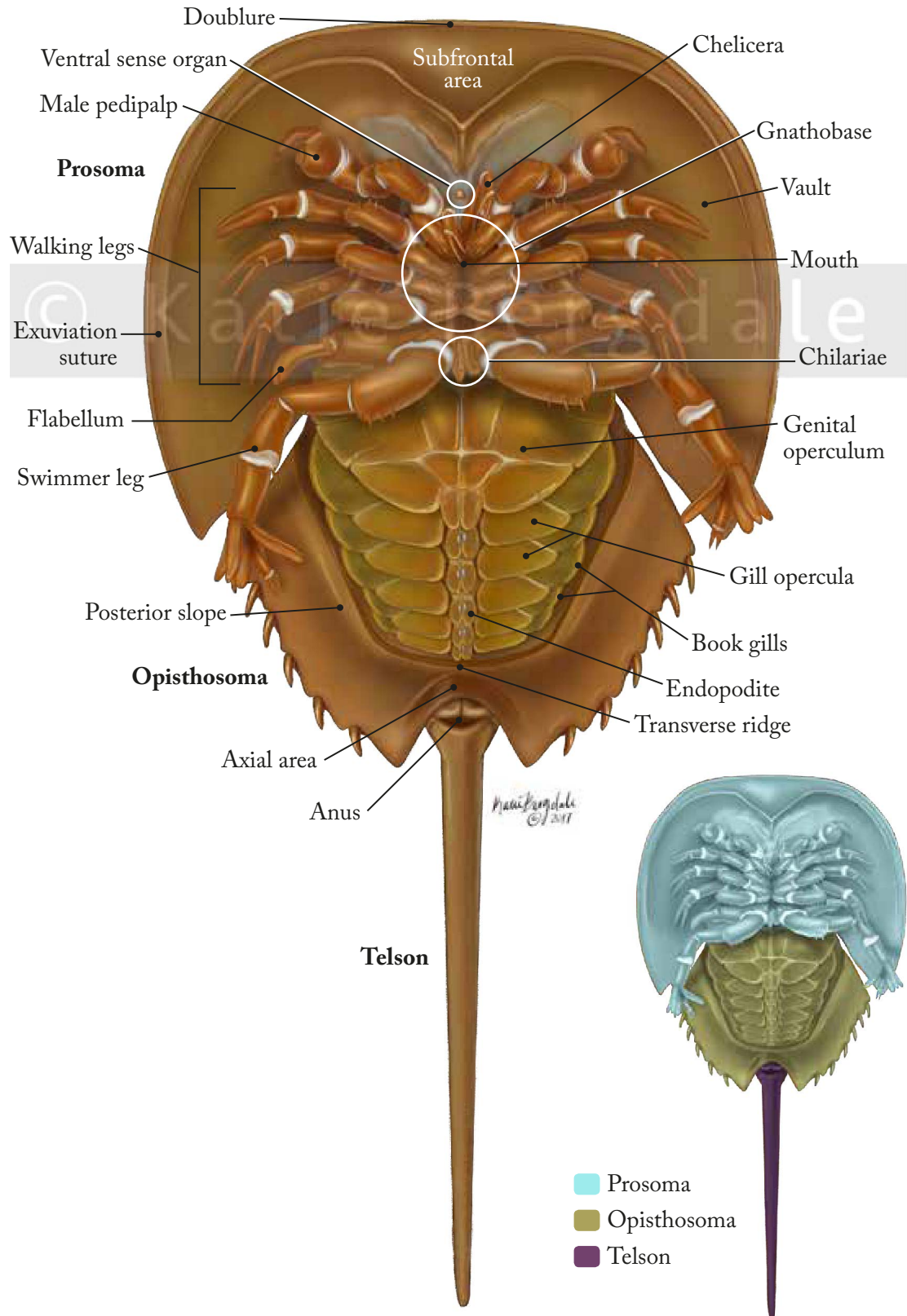


FIG. 2A: External Anatomy: Ventral view  
 FIG. 2B: Segments of the Body: Ventral view

## MATERIALS & METHODS

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### Background Research and Review

The first phase of this project required several meetings with the following content experts: Jill Arnold, MS, MLS (ASCP)<sup>CM</sup>; Brent Whitaker, MS, DVM; and Catherine (Kat) Hadfield, MA, VetMB, MRCVS, Dipl ECZM, Dipl ACZM; all employees at the National Aquarium. Discussions included project objectives, intended audience, and anticipated outcomes for this project. Interaction and observation of specimens was completed to gain familiarity with horseshoe crab anatomy and health assessment techniques currently used at the National Aquarium. A literature review was completed to gain sufficient knowledge of anatomy and physiology of horseshoe crabs. An illustration outline was created and approved by all content experts and faculty advisor, Tim Phelps, MA, FAMI, which listed all required topics with corresponding illustrations and planned outcomes (Appendix C). This outline provided a descriptive of all labels, desired style and media for each illustration, and the overall plan for the project. A trip to the Marine Biological Laboratory (MBL) in Woods Hole, MA was coordinated with Amy Hancock-Ronemus, VMD, the lab animal veterinarian at MBL.

### Marine Biological Laboratory

A week-long trip was taken in January 2017 to the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts because it has been the center of *Limulus* research for over a century (Shuster, et. al., 2003). MBL houses many of their own research specimens at the Marine Resources Center (MRC), where observation and gross dissection are performed at their necropsy lab. This trip provided the opportunity to learn about the species' external and internal anatomy, behavior, and pathology. Time at MBL was divided between performing necropsies, observing normal horseshoe crab behavior, sketching from dead and live specimens, refining sketches and layouts of illustrations, photographing specimens using a Cannon EOS rebel SL1 camera, and reviewing literature. The MBL has an extensive and historical library containing many books,

research articles, and illustrations on the horseshoe crab.

### **Specimen Handling**

Four total necropsies, three males and one female, were performed. One specimen was obtained in October 2016 from the National Aquarium in Baltimore, and the necropsy was performed the same day in the Aquarium's necropsy lab. The specimen had died earlier that day from natural causes. The other three specimens were obtained from MBL during the visit, and the necropsies were performed at the MRC's necropsy facility. Two of the specimens were euthanized immediately prior to dissection and the other was found dead in the MRC animal holding tanks. The euthanasia protocol consists of injecting 3 mL of eugenol, or clove oil through the arthrodial membrane, directly into the heart. The specimen was left for 15-20 minutes and until completely unresponsive. The remains were disposed of per MBL protocol following the dissection.

### **Gross Dissection**

Various techniques and approaches were performed during the dissection process to learn which approach led to the best preservation of anatomy. The main question tested was that of the initial approach: to begin the dissection dorsally or ventrally. The successful steps of each approach are described in the following section. Extreme care was taken during each dissection to preserve fragile anatomy. Basic necropsy instruments used included heavy duty scissors, blunt scissors, and forceps. All necropsies were executed in a proper lab with appropriate lighting, drainage, and ventilation (Fig. 3). Figures describing these approaches are displayed together in the Results section entitled *Necropsy by Ventral Approach* and *Necropsy by Dorsal Approach*. Each image legend explains the method of performing a necropsy to a novice.



FIG. 3: Dissection Image: Performing a dorsal approach dissection at MRC necropsy lab.

### *Necropsy by Ventral Approach*

Ventral dissection began by making a hole with blunt scissors in the soft bluish tissue on the ventral prosoma anterior to the chelicerae (Fig. 5). The following steps were taken to begin the removal of the carapace from the internal anatomy to allow better observation of viscera specimen. The initial cut was carefully continued along the entire inner surface of the prosoma, only 1 cm medial, to minimize the removal of the underlying orange tissue (dashed line, Fig. 5). This orange tissue is comprised of hepatopancreas dorsally and gonadal tissue laterally and ventrally, however these tissues are interdigitated and indistinguishable (pers. comm. Roxanna Smolowitz). Next, the inner carapace was lifted and the interdigitated hepatopancreas and gonadal tissue was separated from both the dorsal and ventral carapace using blunt dissection with a finger to avoid damaging the tissue (Fig. 6). Heavy duty scissors were used to cut the thick triangular edge of the prosoma known as the genal angle. This cut was continued around the entire outer edge of the prosoma to completely remove the subfrontal area, the flat edge of the prosoma (option A, Fig. 7). If the carapace was too dense to cut at the genal

angle, a common occurrence, a cut was made approximately five to seven centimeters anterior at a less dense section of carapace (option B, Fig. 7).

The leg muscles extend through the interdigitated hepatopancreas and gonadal tissue to the dorsal carapace. These leg muscle attachments connecting to the ventral and dorsal carapace must be cut to free the internal anatomy, specifically the interdigitated hepatopancreas and gonadal tissue. First the leg muscles' attachments were cut as close to the dorsal prosoma to preserve the heart and pericardial sac (Fig. 8). Next the leg muscles were cut from the ventral carapace, completely separating the interdigitated hepatopancreas and gonadal tissue from the carapace (Fig. 9). An inset was included in Fig. 9 to illustrate the dissection of a gravid female, or a female containing eggs. Next, a circular cut was made lateral to the mouth through the promesosternite, the ventral membrane that surrounds the mouth, by pushing the gnathobase laterally to expose the mouth (dashed line, Fig. 10). By cutting through the promesosternite, the mouth was preserved, protecting the immediate underlying anatomy: the excretory system, arterial ring and brain. The mouth serves as an important landmark as well once the legs and gnathobase have been removed.

The removal of the ventral carapace and legs exposes the excretory system. The coxal glands lie within musculature of the coxa segments of the second through fifth legs (Fig. 11)(Shuster, et. al., 2003). The excretory system is extremely delicate and difficult to preserve during a necropsy using a ventral approach. Dissection was continued by gently peeling away the excretory system to uncover the arterial ring, which lies immediately dorsal. The brain is encased within the arterial ring and together these structures form a collar around the mouth (Fig. 12). The brain and nervous system are almost entirely enclosed within the arterial system. Some nerves break through the arterial system to additionally innervate important structures such as the appendages or eyes.

The genital operculum was lifted to view the genital pores (Fig. 13). Each sex has

uniquely colored and shaped genital pores. Using blunt scissors, a cut was cautiously made in the midline of the genital operculum and through the next two gill opercula to expose the underlying vascular anatomy that feeds the book gills (dashed line, Fig. 13). The book gills were cut from their attachments as closely to the opisthosoma as possible (Fig. 14: step 1). Blunt scissors were used to puncture two holes in the ventral membrane under the last gill opercula, and two cuts were made on either side of the posterior opisthosoma to the telson (Fig. 14: steps 2-3). Then, a cut was made across the posterior opisthosoma to connect the two previous cuts, and thus, a piece of carapace was removed exposing the underlying anatomy (Fig. 14: step 4). A final cut was made to completely separate the ligaments and intestines from the carapace. The viscera specimen was removed from the carapace.

The specimen was carefully turned dorsally to observe the heart and pericardial sac (Fig. 15). The heart and pericardial sac are difficult to distinguish from each other due to the similar colors and textures. The heart resides inside of the pericardial sac but is shown in Fig. 15 diagrammatically for clarification of the borders of each structure. Vascular structures observed include frontal artery, ostia, aortic valve, lateral arteries, collateral arteries, branchio-cardiac arteries, and superior abdominal artery.

The viscera specimen was turned back over to expose the ventral side. Beginning at the mouth, a cut was made through the arterial ring and brain and continued anteriorly to open the esophagus. This cut was continued to open the crop but ended at the opening of the gizzard (Fig. 16). The gizzard was first gently exposed from the surrounding tissue and the specimen was turned over to view the dorsal side (Fig. 17). Using a blunt scissors, the gizzard was cut open to the pyloric valve. The heart and pericardial sac were cut and peeled away to expose the intestine, and the glandular hepatic ceca, or connections from the midgut to the hepatopancreas were observed (Fig. 18).



*Necropsy by Dorsal Approach*

Dissection began with a cut perpendicular to the carapace edge to create an entry point and continued around the entire edge of the prosoma, positioned approximately 1 cm medial to the prosomal edge (Fig. 19: 1). This initial cut was easier to make anterior to the genal angle. A cut was then made along the midsagittal plane or midline of the carapace up to the arthroal membrane (Fig. 19: 2). Beginning at the edge of the prosoma, parallel with the hinge joint, a cut was made to the arthroal membrane (Fig. 19: 3). This was repeated bilaterally, and the prosoma was disconnected from the leg muscle attachments and peeled off (Fig. 19: 4). This provided an open view of the anterior half of the heart and pericardial sac. Next, cuts were made through the opisthosomal carapace on either side of the arthroal membrane to the telson along the longitudinal furrows, lateral to the entapophyseal pits, or the attachment points that connect the book gills to the opisthosoma (Fig. 19: 5 - 6). A cut through the midline keel at the anterior opisthosoma was made, preserving the arthroal membrane. Next, a cut through the posterior midline keel was made to connect cuts 5 and 6 (Fig. 19: 8). The opisthosomal appendage attachments were cut from the opisthosoma and this piece of carapace was removed. The opisthosoma carapace was removed from the internal anatomy. Unlike in the ventral approach, the ventral carapace remained on the specimen.

The heart, pericardial sac, vascular anatomy, leg muscle attachments, and the interdigitated hepatopancreas and gonadal tissue were observed. By palpating the orange interdigitated hepatopancreas and gonadal tissue, the large gizzard was found and uncovered. By removing the heart and pericardial sac, the intestine was exposed. Next the gizzard was cut open, exposing the rough denticles within; this cut was continued ventral through the esophagus and cartilage shelf, or endosternite, which sits directly ventral to the intestine (Fig. 18). This exposes the arterial ring that houses the brain. The arterial ring was peeled away to uncover the excretory system.

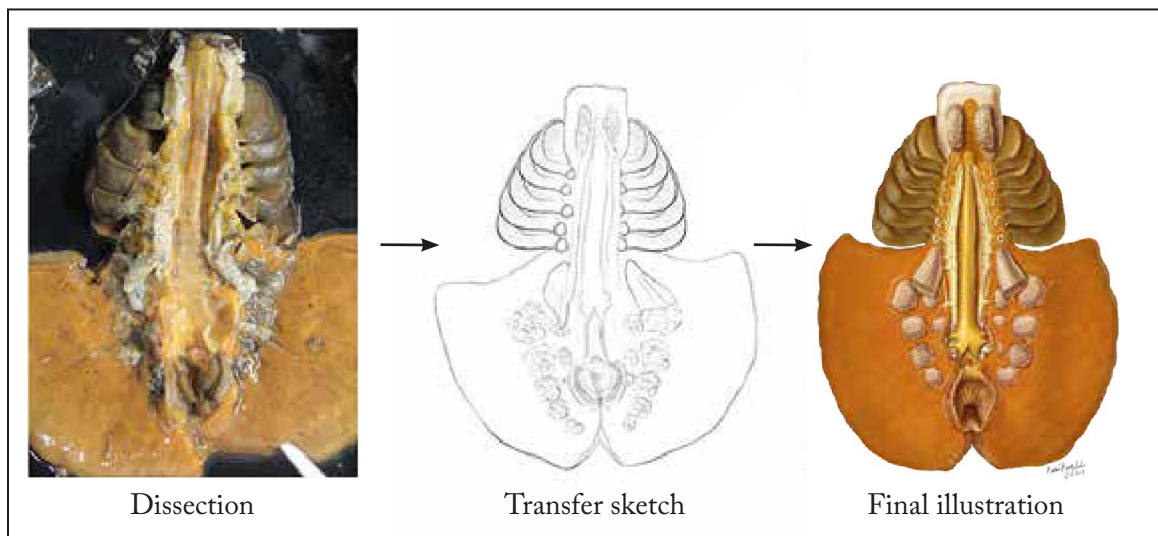
### Illustration Technique

Once background research was completed, each illustration began with thumbnail sketches to plan and organize the composition based on the illustration outline (Table 1, Appendix C). Storyboards were created to finalize layouts, text, and base art, or reusable illustrations. The process of storyboarding the necropsy illustrations revealed the level of complexity and number of illustrations and labels necessary to effectively explain each step. Due to the large number of illustrations this project required in a relatively short amount of time, reusable base art was planned to save time and provide consistency throughout the project. Figures 1 and 2 seen in the External Anatomy section were created first in Adobe® Photoshop CC 2017 as base art for most of the proceeding illustrations. These illustrations were created using multiple layers and masks in Photoshop to allow for future manipulation. For example, the illustration of the carapace in Figure 1A was separated into three sections within Photoshop, following the same main segments of the horseshoe crab body: prosoma, opisthosoma, and telson. Each leg segment and gill opercula were drawn on individual layers, allowing individual layers to be shown or hidden depending on the needs of each illustration. The genital and first gill operculum were completely drawn then masked, or partially hidden depending on the desired outcome. For example, the anterior section of the genital operculum in Figure 1 is hidden behind the legs, so its layer was partially masked, but the entire genital operculum in Figure 11 was visible with the legs removed, so it is unmasked or shown. Another use of base art can be appreciated in the gill opercula; the first was created and replicated for the following gill opercula. Changes in size and additional details created diversity, but this process saved time and created consistency.

Final sketches were created from direct observation and photographs of living and dissected specimens as well as reference literature (workflow example, Fig. 4). The final transfer sketches were finalized in Procreate® for Apple® iPad Pro, a drawing application,



to create clean sketches on a transparent background. They were then transferred into Photoshop for final rendering. Each transfer sketch was evaluated and approved by all project advisors (Jill Arnold, Brent Whitaker, Kat Hadfield, and Tim Phelps) to ensure anatomic accuracy and clarity. For each illustration, digital friskets (a solid shape in which paint and texture can be applied without exceeding the borders) were created and an airbrush technique was used to create a realistic aesthetic. In the necropsy series, each illustration began with the ventral or dorsal external anatomy illustration as the base art, and new layers were added on top. For Figures 20 and 21, Adobe® Illustrator CC 2017 was used to create clean diagrams. Then Figures 20 and 21 were transferred into Photoshop for additional enhancement including tone. Each completed illustration was exported as a .PNG file and placed into Adobe® InDesign CC 2017 to add labels and figure legends.



**FIG. 4:** Illustration Workflow: From dissection photograph to transfer sketch to final illustration.

## RESULTS

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This study provides multiple detailed illustrations relating to the biology of the horseshoe crab. Figures 1 and 2 describe the notable external anatomy of the horseshoe crab. For a description of external anatomy, refer to the text description entitled *External Anatomy* within the Introduction section.

### **Necropsy Guide**

A realistic guide was created to explain the proper method for performing a necropsy on the horseshoe crab using two different starting approaches (Fig. 5 through Fig. 18, *Necropsy by Ventral Approach*; Fig. 19, *Necropsy by Dorsal Approach*). Both approaches illustrate step by step dissection of a horseshoe crab in a clear, realistic way. This series describes the necropsy procedure in a comprehensive, anatomically faithful, and sequential manner. The images provides clear, accurate illustrations and descriptions of the steps required to preserve important anatomical structures, which can be easily damaged or destroyed. Each illustration also includes labels of important anatomical features seen during that step. The observed anatomical systems include: excretory, digestive, circulatory, nervous, and reproductive.

These illustrations guide the audience to complete a successful necropsy and teach the complex anatomy of the horseshoe crab. An understanding of normal biology allows for proper diagnosis of abnormalities or diseases, contributing to better conservation strategies and proper health care.

The following is an instructional description of the steps for completing dissection by ventral and dorsal approach with explanation of supporting elements (Fig. 5 through 19).

#### ***Ventral Approach***

Step 1 (Fig. 5): To begin, pierce the ventral carapace in the section of bluish soft tissue using blunt scissors. Then, cut along the dashed line through the inner shell layer

only. It was discovered that this location proves most accessible when making the initial cut due to the soft tissue present. A dashed line with arrows indicates the path on which to cut.

Step 2 (Fig. 6): Carefully peel back the ventral carapace to separate it from the interdigitated hepatopancreas and gonadal tissue. This separation is important in preventing damage to the interdigitated hepatopancreas and gonadal tissue in future dissection steps. Blunt dissection with a finger works best to separate this delicate tissue from the carapace. An arrow indicates the ideal path in which to pull the carapace.

Step 3 (Fig. 7): (A) Using a heavy duty scissors, cut the thick triangular end of the prosoma known as the genal angle and continue to cut the outer edge of the prosoma along the same path described in Step 1. (B) If the carapace is too dense to cut at the genal angle, cut 5 cm anterior. Then, continue along the outer edge of the prosoma. The second option (B) is included because one specimen encountered had an extremely tough shell which was impenetrable at the genal angle. This led to the addition of an option more anterior, where the carapace is less dense. The use of dashed lines with arrows shows the direction in which to make the cut.

Step 4 (Fig. 8): Separate the interdigitated hepatopancreas and gonadal tissue from the dorsal carapace by carefully cutting all the leg muscle attachments off of the dorsal carapace up to the hinge joint. To avoid damaging the heart, cut the muscle attachments as closely as possible to the dorsal carapace. Hands and instruments are included within this series to provide realistic guidance. It was discovered when dissecting a male that the interdigitating hepatopancreas and gonadal tissue are indistinguishable but dorsally are mostly comprised of hepatopancreas, while ventrally and laterally primarily gonadal tissue (pers. comm. Roxanna Smolowitz).

Step 5 (Fig. 9): Cut the muscle attachments connecting the legs to the ventral carapace to completely separate the carapace from the interdigitated hepatopancreas

and gonadal tissue. Again, cut as closely to the ventral carapace as possible to preserve underlying anatomy. Repeat on the opposite side. As shown in the inset, when dissecting a gravid female, thousands of eggs are present, completely covering the ventral surface of the gonadal tissue.

Step 6 (Fig. 10): Make a circular cut around the mouth through the promesosternite, the ventral membrane that surrounds the mouth, by pushing the gnathobase laterally. By cutting through the promesosternite instead of the mouth, it preserves the immediate underlying anatomy: excretory system, arterial ring and brain. It is important to preserve the mouth because it serves as an excellent starting reference when exposing the digestive system. An inset was included to provide the viewer with more detail.

Step 7 (Fig. 11): Observe the excretory system if it is still intact. The tissues are extremely fragile and difficult to preserve during a ventral approach necropsy. Continue dissection by gently exposing the arterial ring and brain, located directly dorsal to the excretory system. The structures that make up the excretory system were enlarged as an inset and labeled for clarification.

Step 8 (Fig. 12): Observe the arterial ring. The brain resides entirely inside of the arterial ring and most nerves reside within the arterial system. The inset shows the brain and nerves within the arterial ring, using instructional color to distinguish between the structures. Note the nerves penetrating through the arterial ring are difficult to visualize during a dissection and only appreciated in the inset.

Step 9 (Fig. 13): Lift the genital operculum to view the subtle differences between the genital pores between sexes. Cut the genital operculum and two posterior gill opercula at the midline to expose the underlying vascular anatomy. Due to the small size of the genital pores on the main illustration, each sex's pores were enlarged and

positioned together for comparison. The dash line at the midline of the genital and gill opercula indicates where the cut should be made.

Step 10 (Fig. 14): (1) Carefully separate the gills from their attachments to the opisthosoma. (2) Using blunt scissors, make a puncture through the ventral membrane under the most posterior gill operculum and cut through the opisthosoma to the telson. (3) Repeat on other side and remove this piece of carapace. (4) Cut through the ligaments and intestines to remove the viscera from the outer carapace. The numbers alongside the dashed lines indicate the sequence of steps.

Step 11 (Fig. 15): Turn over the viscera specimen to reveal the dorsal surface, and examine the heart and pericardial sac. While the heart resides within the pericardium, it is shown here diagrammatically for clarification of the borders of each structure. Small positional icons are introduced where the specimen no longer possesses a carapace. This indicates the position of the specimen during the dissection.

Step 12 (Fig. 16): Turn over the specimen to expose the ventral side. (1) Starting at the opening of the mouth, cut anteriorly through the arterial ring, brain, and (2) the esophagus up to the opening of the gizzard. Using blunt dissection, gently expose the gizzard from the surrounding tissue to view the external structure. It can be observed when opening the digestive tract most accurately when the initial cut begins at the mouth. The esophagus can be used as a guide to locate the gizzard. An inset describes the two sub-steps examined in Step 12.

Step 13 (Fig. 17): Turn over the specimen to view the dorsal side again. Cut open the gizzard to expose the inner denticles and the pyloric valve. Carefully divide the heart and pericardial sac at the midline to expose the entire intestine.

Step 14 (Fig. 18): Observe the intestine (midgut and hindgut) and the four glandular hepatic ceca or connections from the midgut to the hepatopancreas.

### *Dorsal Approach*

Labels, numbers, dashed lines and arrows, guide the viewer through the dissection. The use of heavy-duty scissors is recommended.

Step 1 (Fig. 19): Make a cut perpendicular to the carapace edge to create an entry point and continue around the entire edge of the prosoma, approximately 1 cm medial to the prosomal edge. This depth best preserves the important underlying anatomy.

Step 2 (Fig. 19): Cut along the midsagittal plane or midline to the arthrodistal membrane, but not through it. The arthrodistal membrane is extremely difficult to cut through and best left attached.

Step 3 (Fig. 19): Begin cutting parallel to the arthrodistal membrane, anterior to the genal angle. Cut up to the arthrodistal membrane.

Step 4 (Fig. 19): Repeat on other side. Cut the dorsal leg muscle attachments, and peel away the prosoma. This exposes the anterior portion of the heart and pericardial sac.

Step 5 (Fig. 19): Cut toward the telson along the longitudinal furrow, lateral to the entapophyseal pits. Note that it can be difficult to start a cut at this part of the carapace.

Step 6 (Fig. 19): Repeat Step 5 on the opposite side.

Step 7 (Fig. 19): Cut through the midline keel at the anterior opisthosoma to preserve the arthrodistal membrane. Note that the heart and pericardial sac can still be adequately observed with the arthrodistal membrane left intact.

Step 8 (Fig. 19): Cut through the midline keel at the posterior end of the opisthosoma to connect cuts 5 and 6. Be sure to cut through the attachments connecting the gill opercula to the opisthosoma, and remove this piece of the carapace.

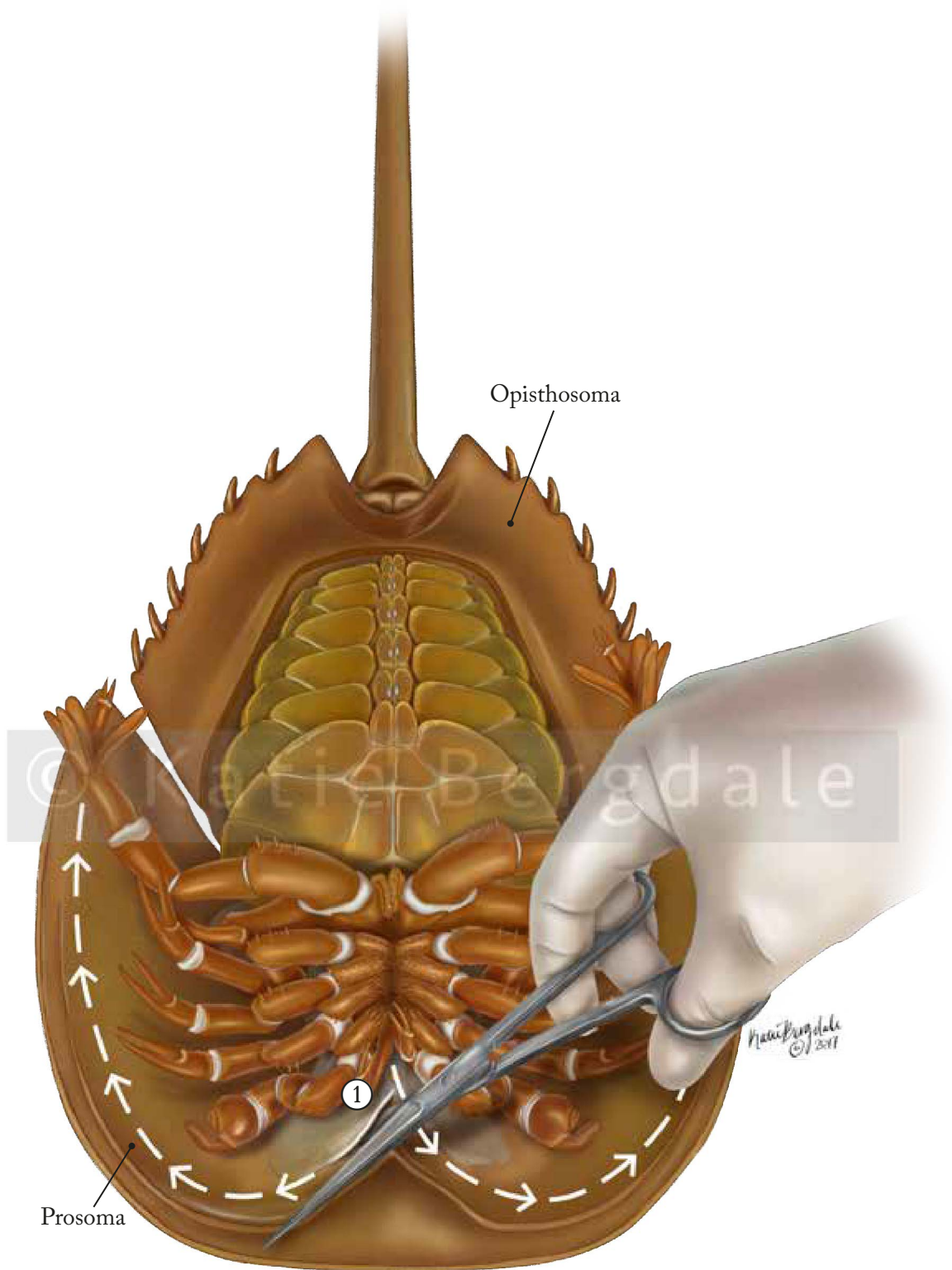
Step 9: Palpate the orange interdigitated hepatopancreas and gonadal tissue to locate the gizzard. Expose the gizzard, intestine, rectum and anus by dividing the

heart and pericardial sac. Note that some hepatopancreas lies between the intestine and pericardial sac.

Step 10: Starting at the gizzard, divide the esophagus and cartilage shelf known as the endosternite (Fig. 18), which sits directly ventral to the intestine. This exposes the arterial ring.

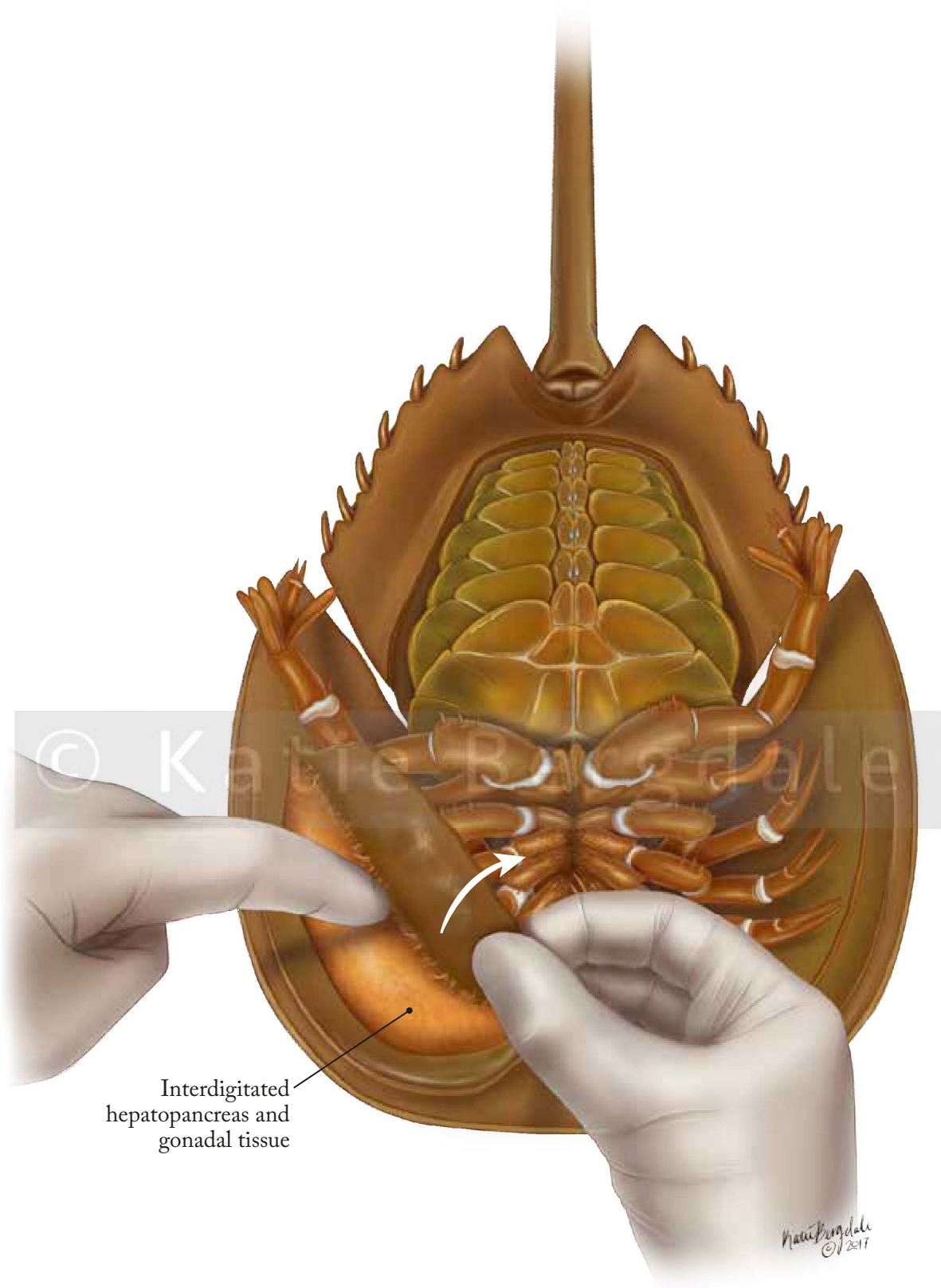
Step 11: Observe the arterial ring which houses the brain. Note that the brain is extremely difficult to distinguish from the arterial ring.

Step 12: Gently remove the arterial ring to uncover the excretory system. The most evident structures of the excretory system are the coxal glands. These glands are a bright reddish color and reside within the musculature in the coxa segments of the legs.

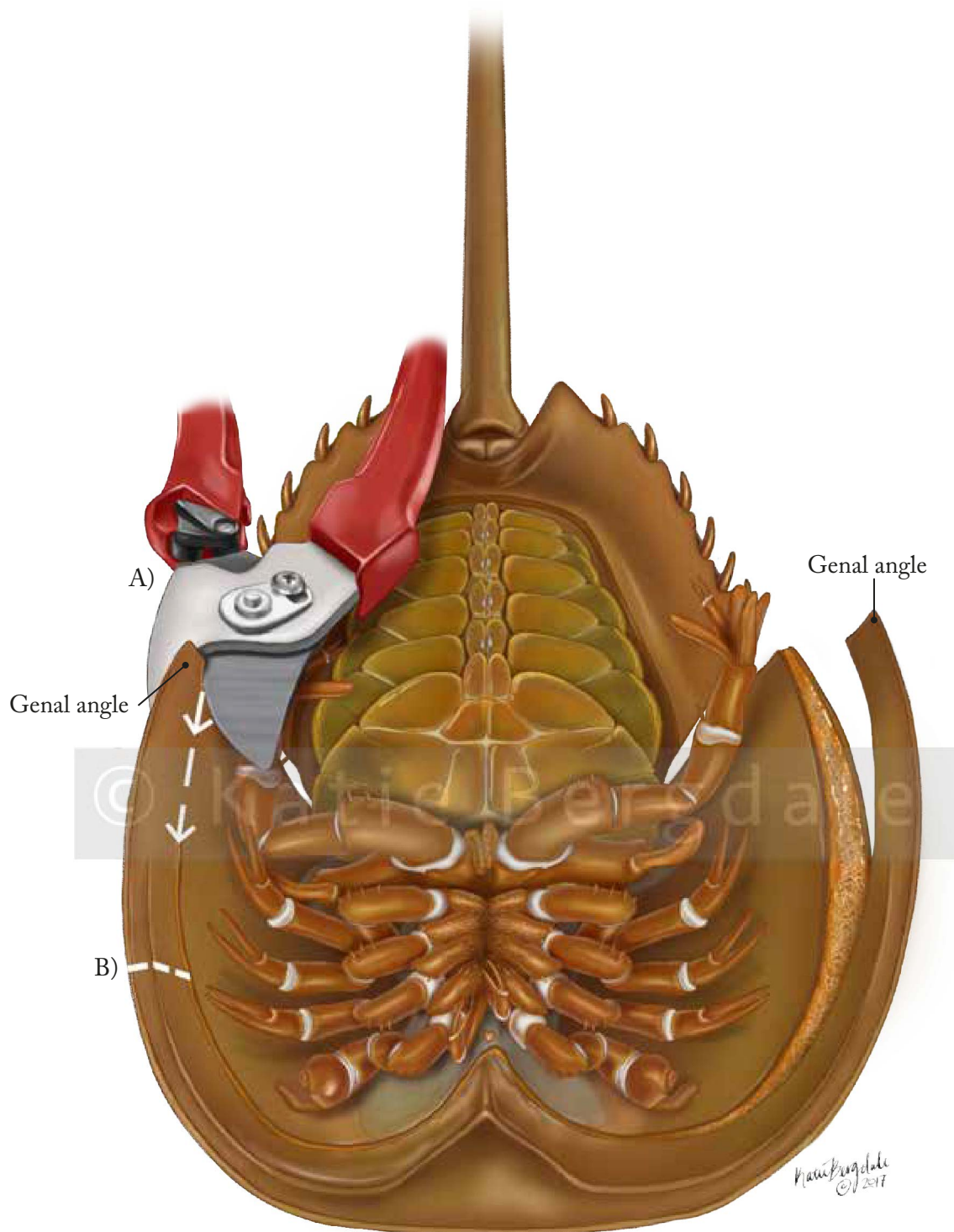


**FIG. 5:** Necropsy by Ventral Approach, Step 1: To begin, pierce the ventral carapace in the area indicated (1) using blunt scissors. Then, cut along the dashed line through the inner shell layer only.

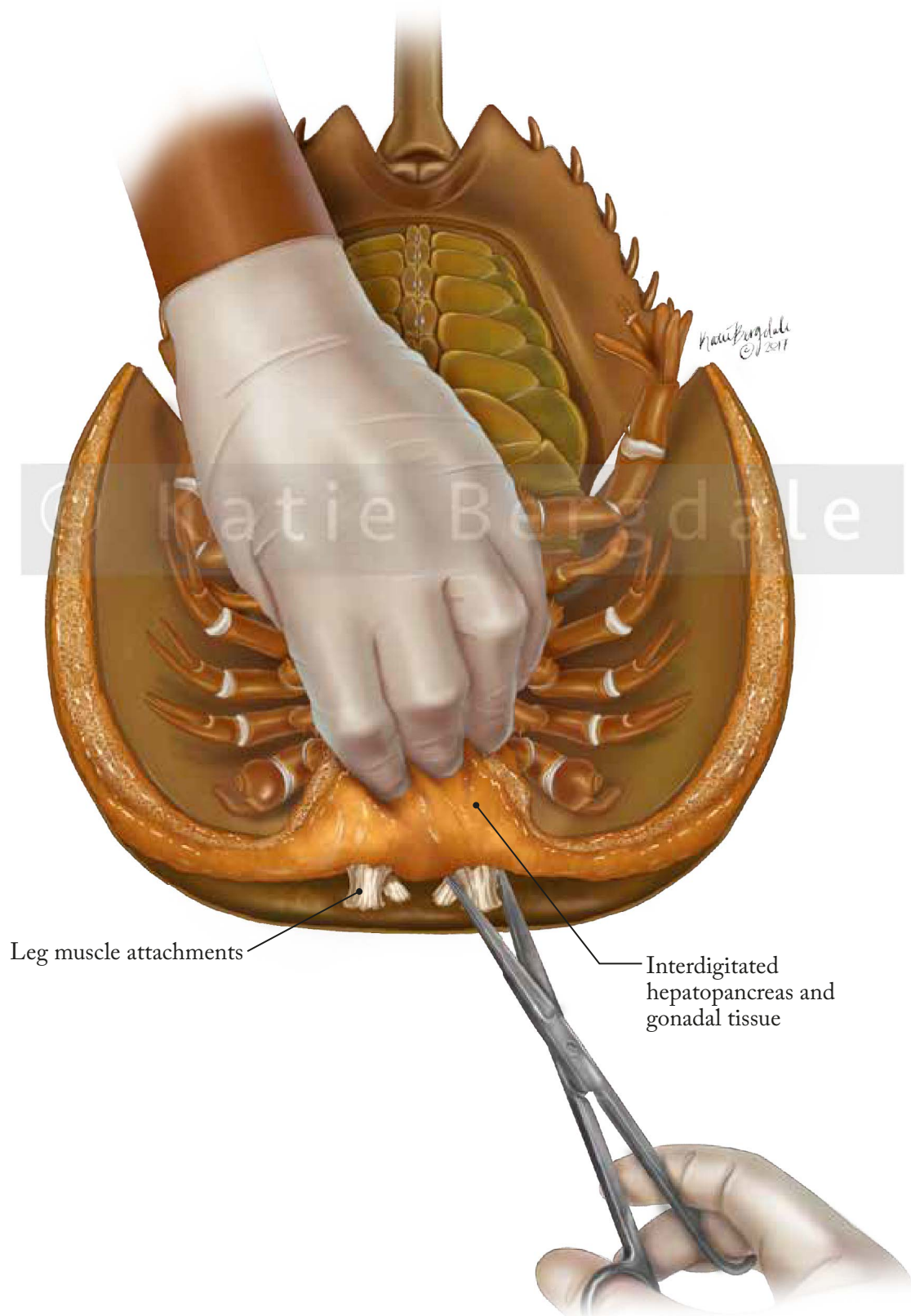




**FIG. 6:** Necropsy by Ventral Approach, Step 2: Carefully peel back the ventral carapace, separating it from the interdigitated hepatopancreas and gonadal tissue using a finger.

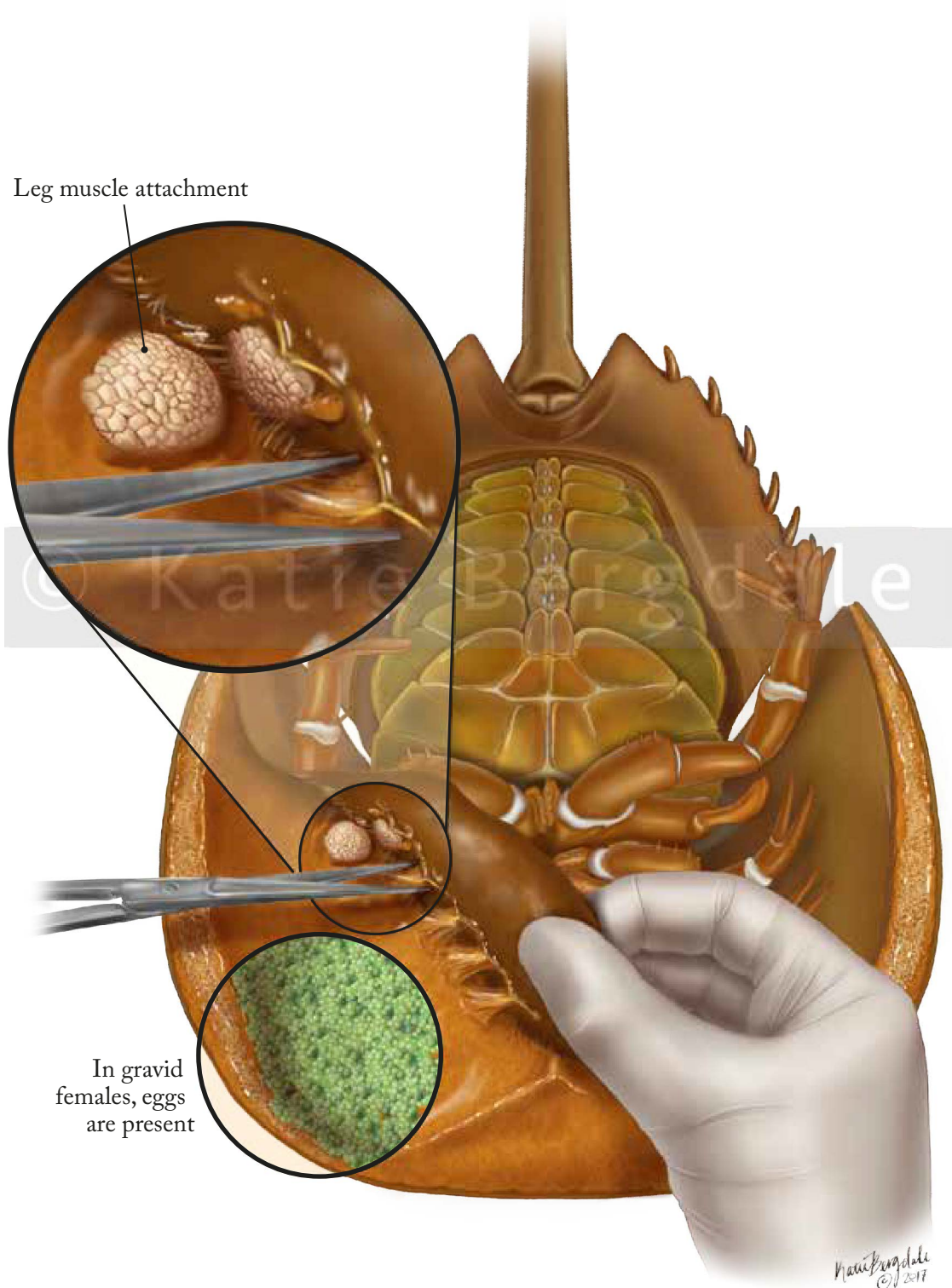


**FIG. 7:** Necropsy by Ventral Approach, Step 3: (A) Using heavy duty scissors, cut the thick triangular end of the prosoma known as the genal angle and continue to cut the outer edge of the prosoma along the same path described in Step 1. (B) If the carapace is too dense to cut at the genal angle, cut 5 cm anterior. Then, continue along the outer edge of the prosoma.

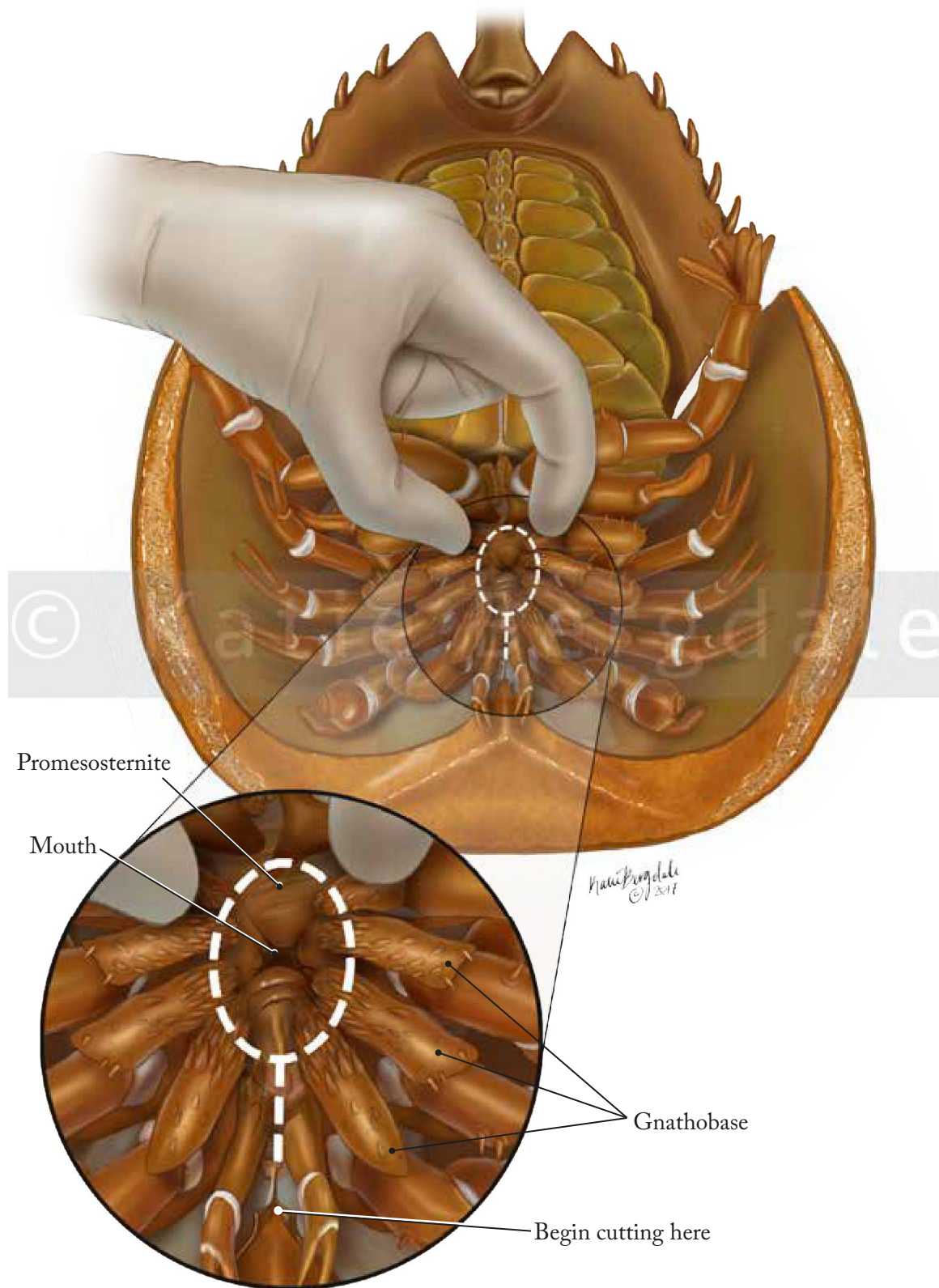


**FIG. 8:** Necropsy by Ventral Approach, Step 4: Separate the interdigitated hepatopancreas and gonadal tissue from the dorsal carapace by carefully cutting all leg muscle attachments off the dorsal carapace up to the hinge joint. Be careful to cut as closely to the dorsal carapace as possible to preserve the heart.

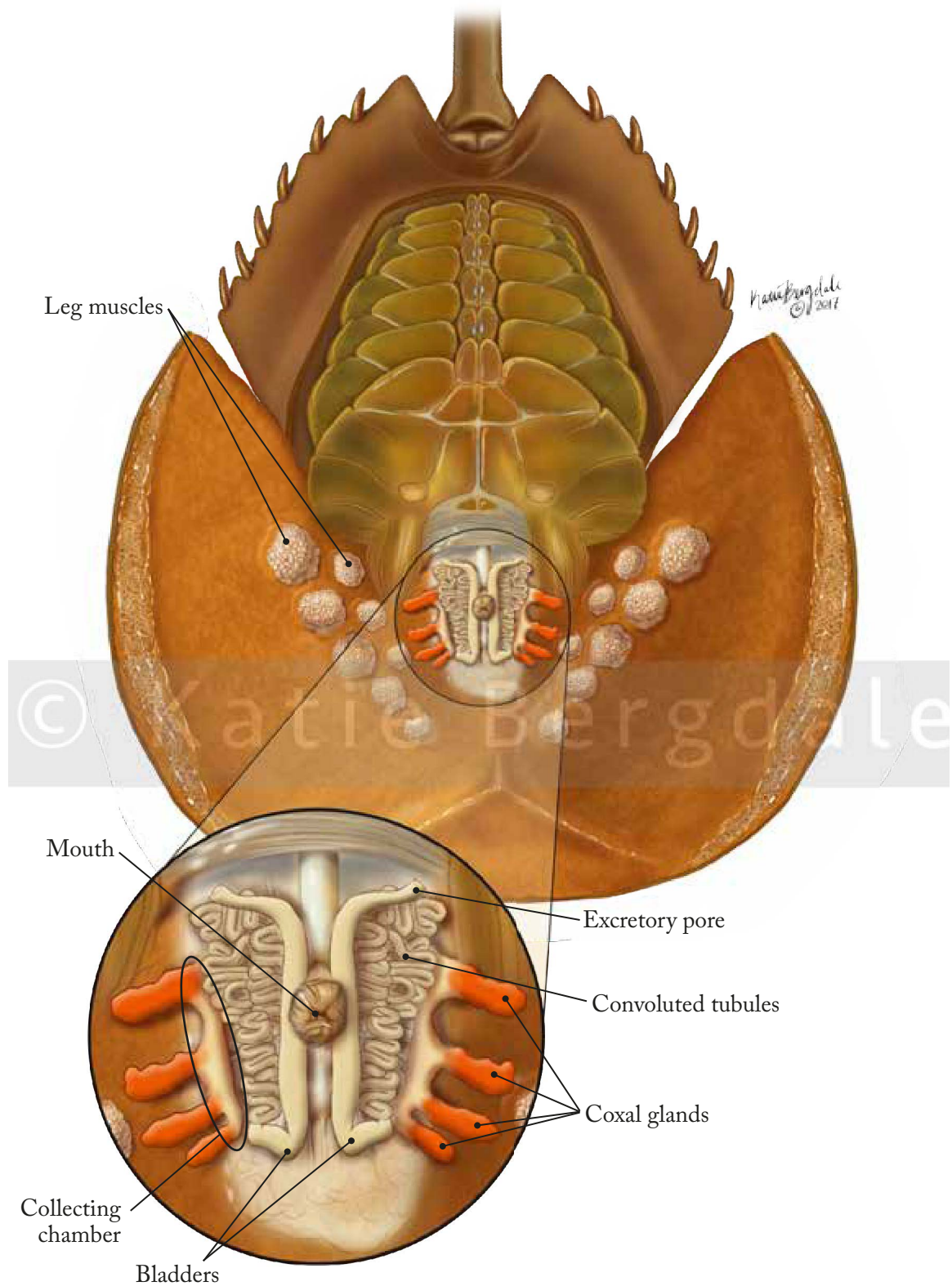




**FIG. 9:** Necropsy by Ventral Approach, Step 5: Cut the leg muscle attachments connected to the ventral carapace to completely separate it from the hepatopancreas and gonadal tissue. Cut as closely to the ventral carapace as possible to preserve underlying anatomy. Repeat on the opposite side. The lower circle inset shows the dense egg production covering the gonadal tissue seen when dissecting a gravid female.

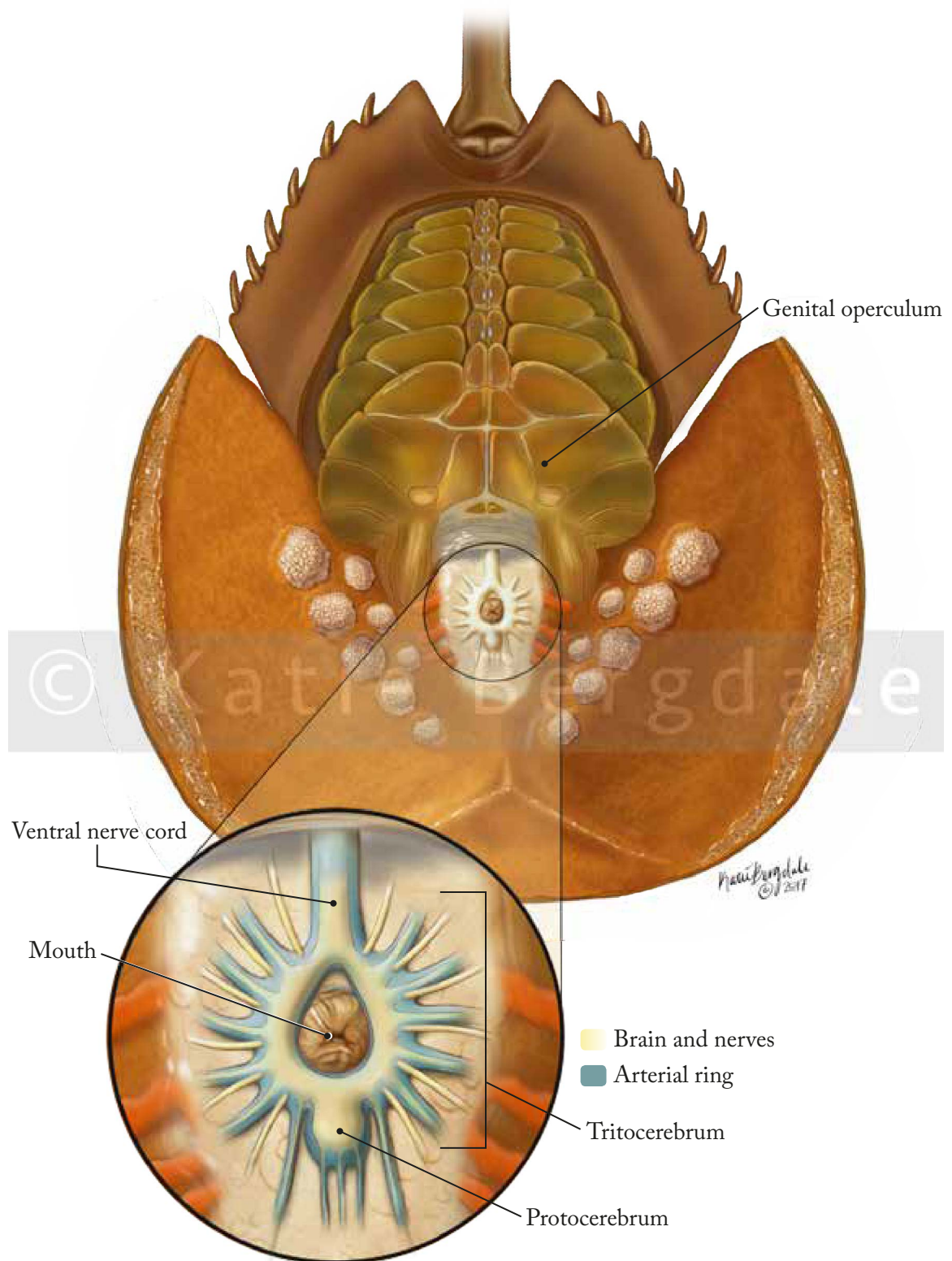


**FIG 10:** Necropsy by Ventral Approach, Step 6: Make a circular cut around the mouth through the promesosternite (the ventral membrane that surrounds the mouth) by pushing the gnathobase laterally. Cutting through the promesosternite instead of the mouth, preserves the immediate underlying anatomy: excretory system, arterial ring and brain.

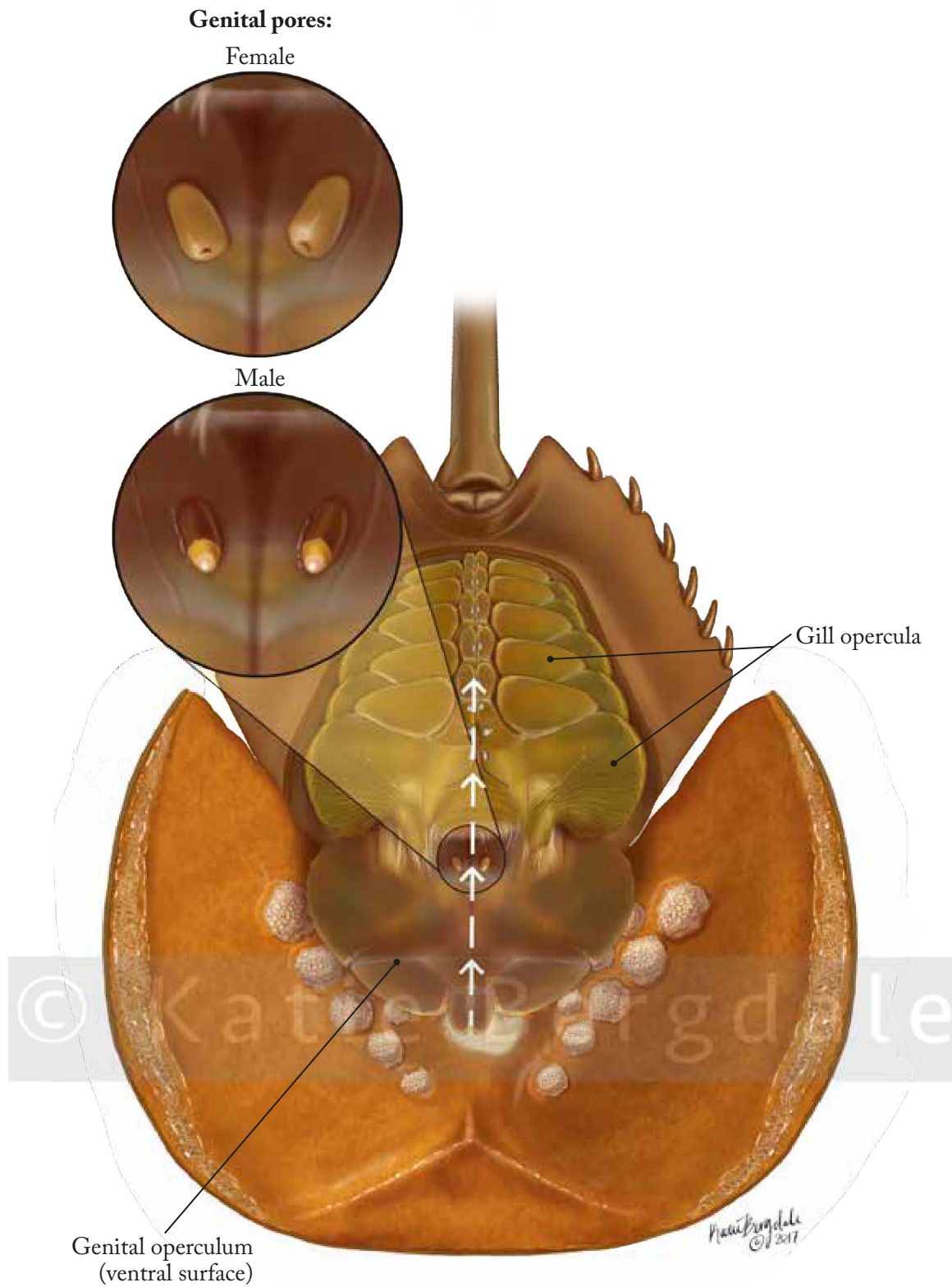


**FIG. 11:** Necropsy by Ventral Approach, Step 7: Observe the excretory system if still intact. The tissues are extremely fragile and difficult to preserve using this ventral approach. Continue dissection by gently exposing the arterial ring and brain located immediately dorsal to the excretory system.



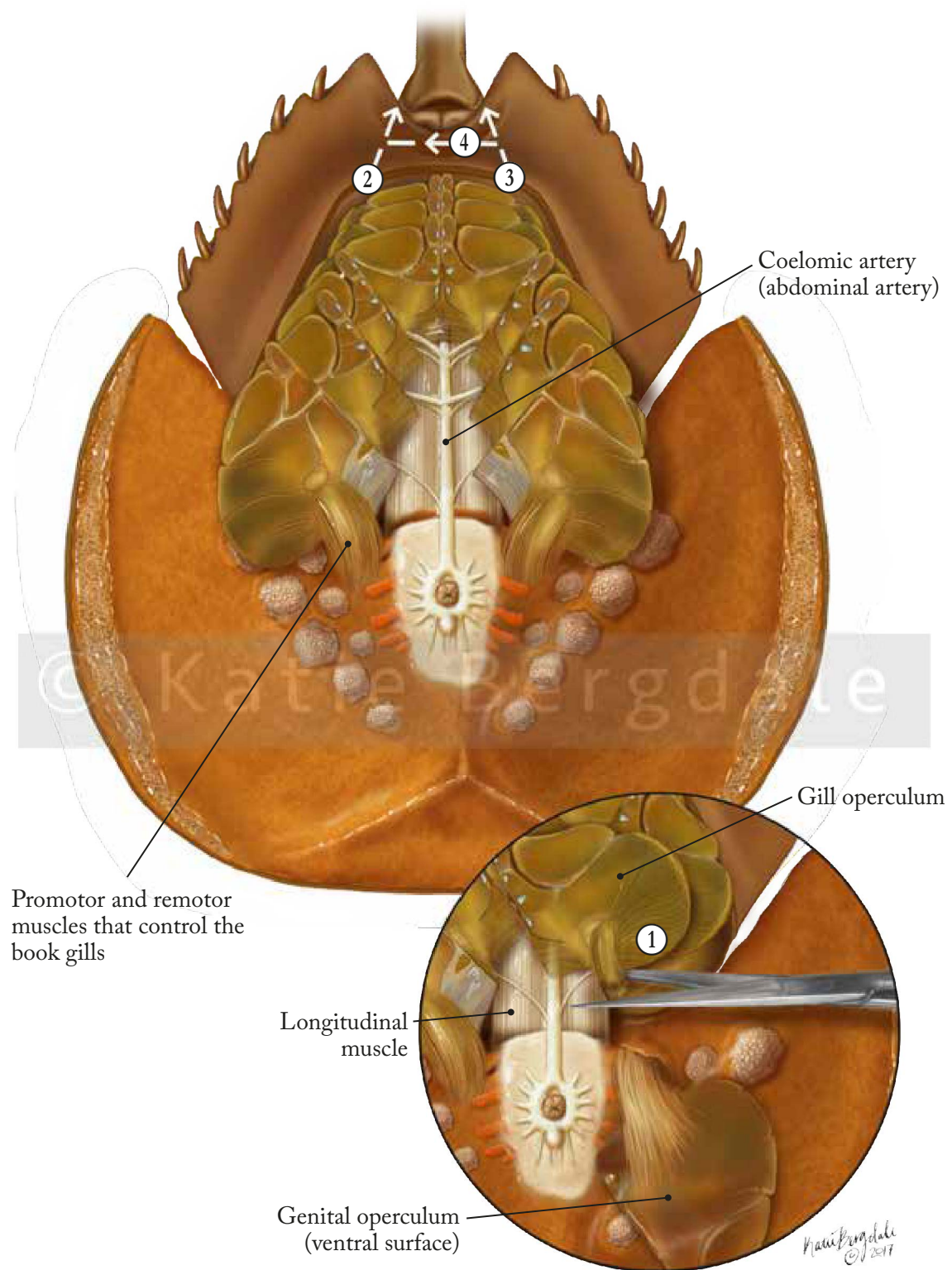


**FIG. 12:** Necropsy by Ventral Approach, Step 8: Observe the arterial ring. The brain resides entirely within of the arterial ring, and most nerves reside inside the arterial system. Note that the nerves penetrating through the arterial ring shown in the close up inset are difficult to visualize during a dissection but shown here for clarification.

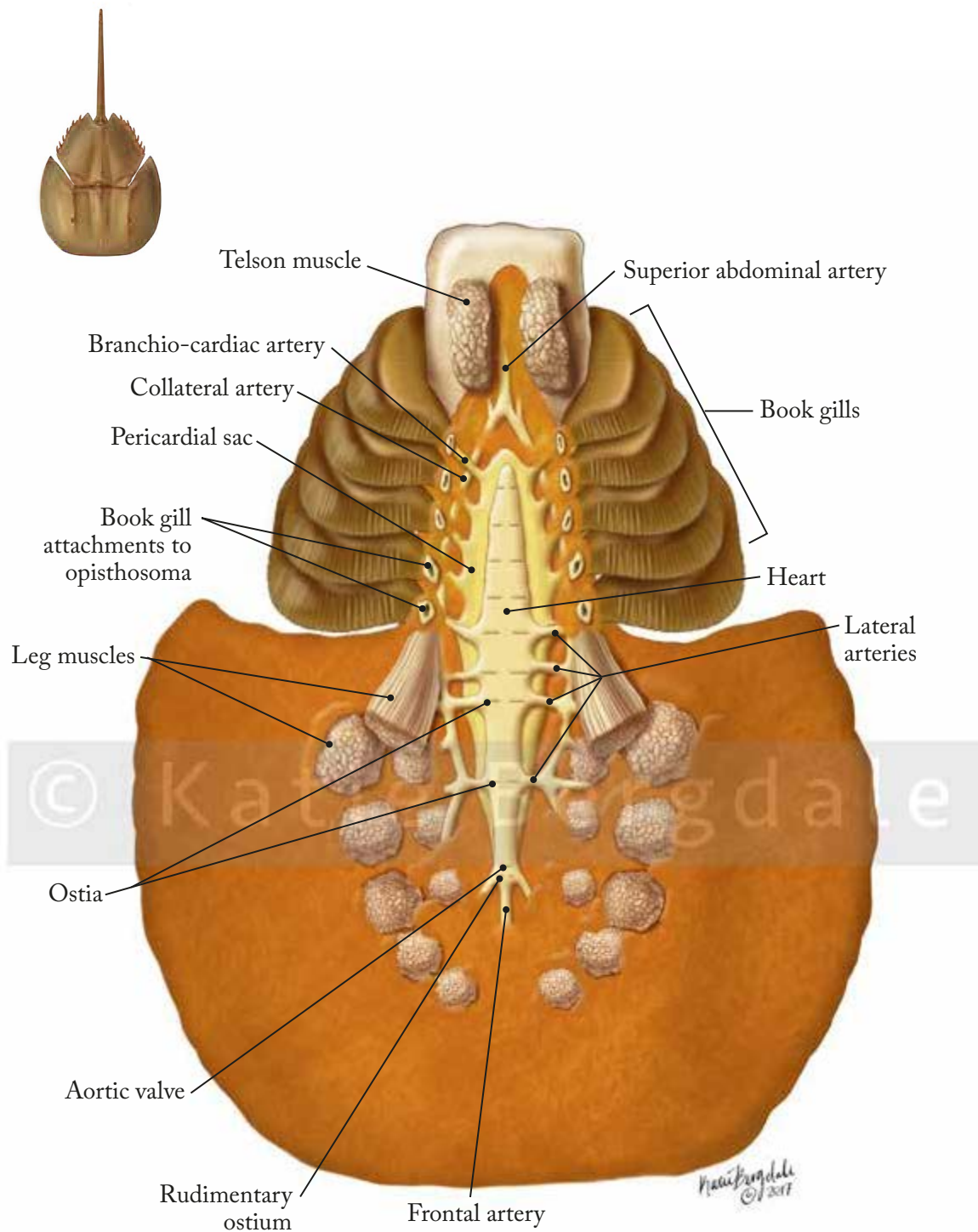


**FIG. 13:** Necropsy by Ventral Approach, Step 9: Lift the genital operculum to view the subtle differences of the genital pores. Cut the genital operculum and two posterior gill opercula at the midline to expose the underlying vascular anatomy.

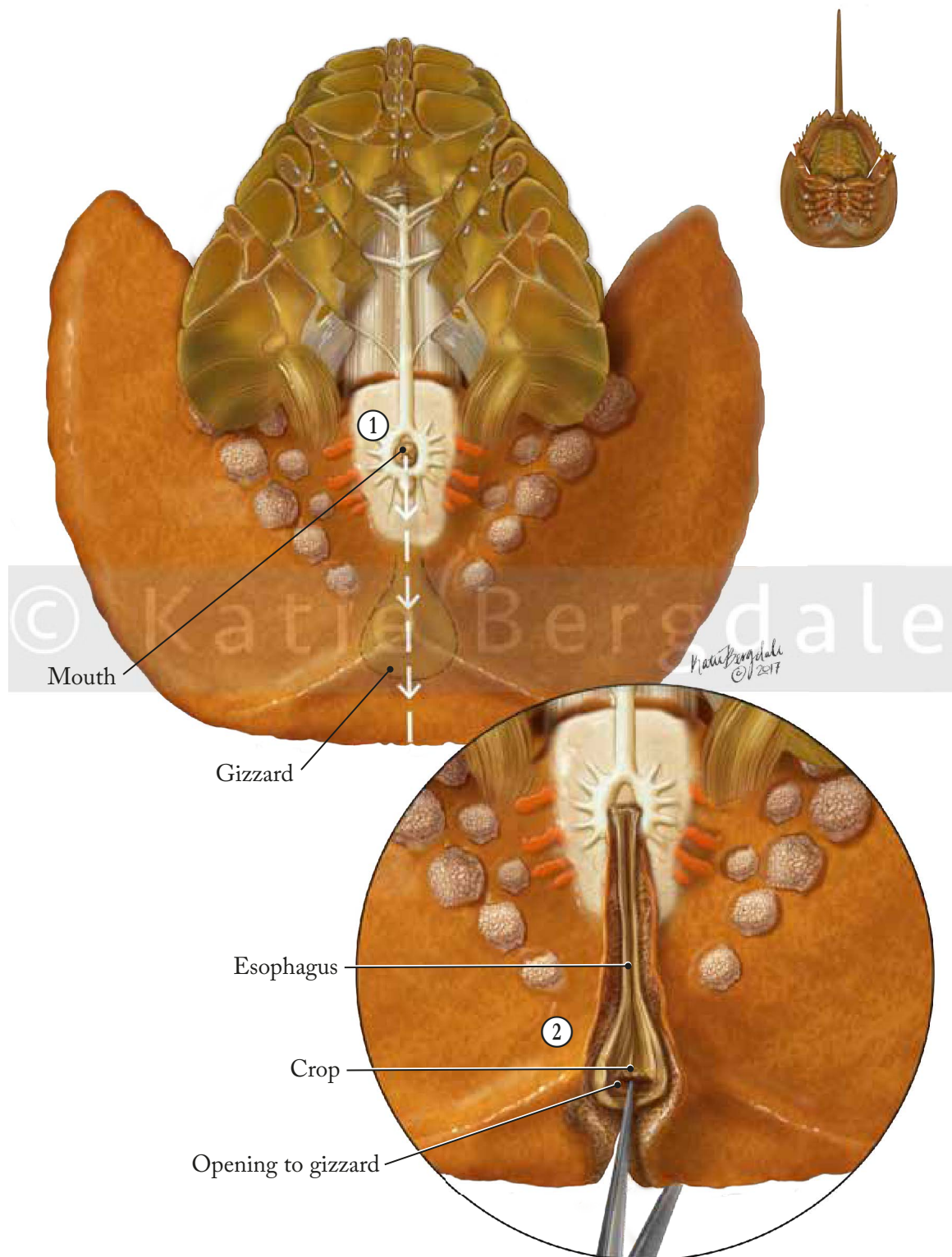




**FIG. 14:** Necropsy by Ventral Approach, Step 10: (1) Carefully separate the gills from their attachments to the opisthosoma. (2) Using blunt scissors, make a puncture through the ventral membrane under the most posterior gill operculum and cut through the opisthosoma to the telson. (3) Repeat on other side and remove this piece of carapace. (4) Cut through the ligaments and intestines to remove the viscera from the outer carapace.

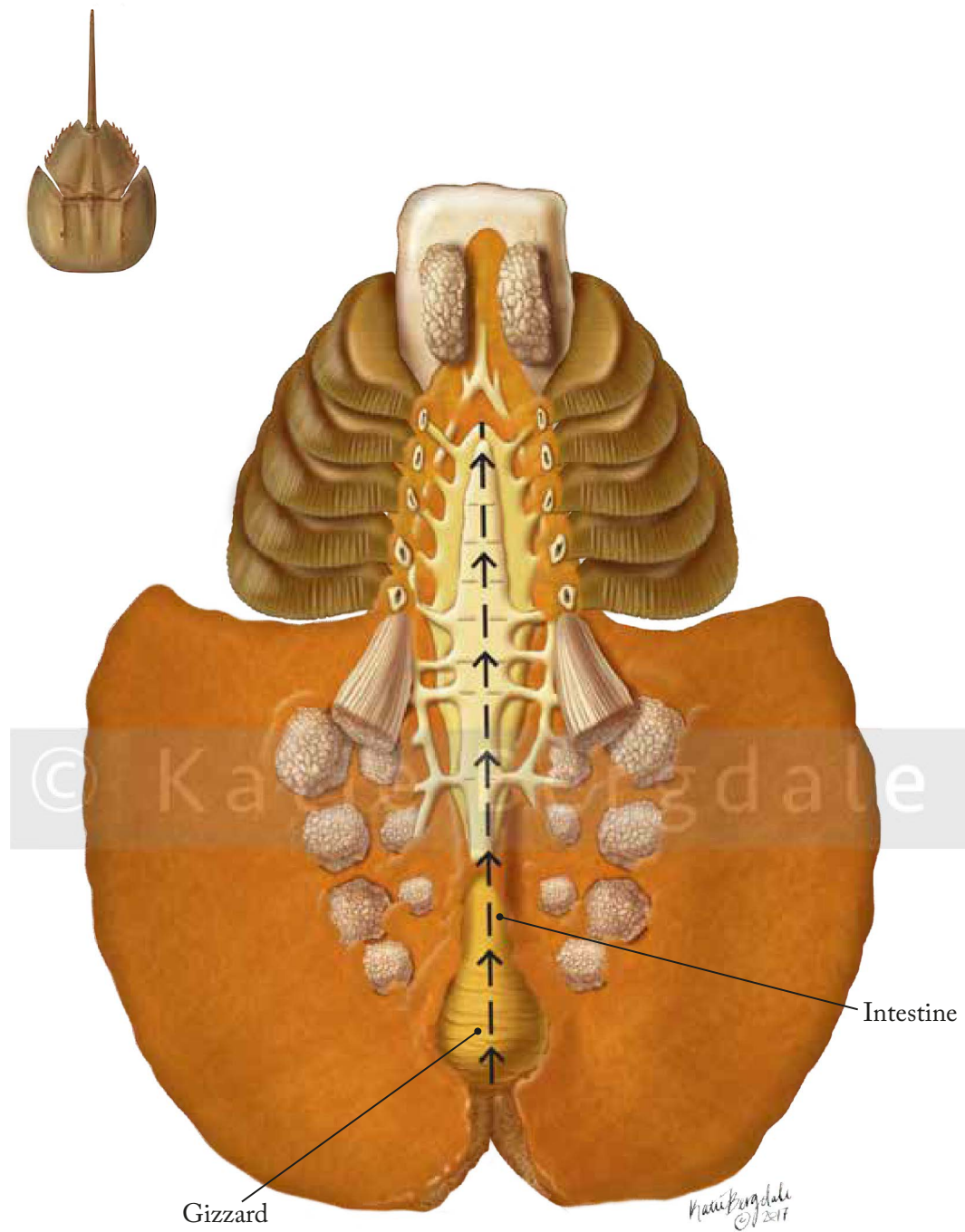


**FIG. 15:** Necropsy by Ventral Approach, Step 11: Turn over the viscera specimen to reveal the dorsal surface, and examine the heart and pericardial sac. While the heart resides within the pericardial sac, it is shown here diagrammatically for clarification of the borders of each structure. (For a detailed diagram of the circulatory system, see Figure 21.)

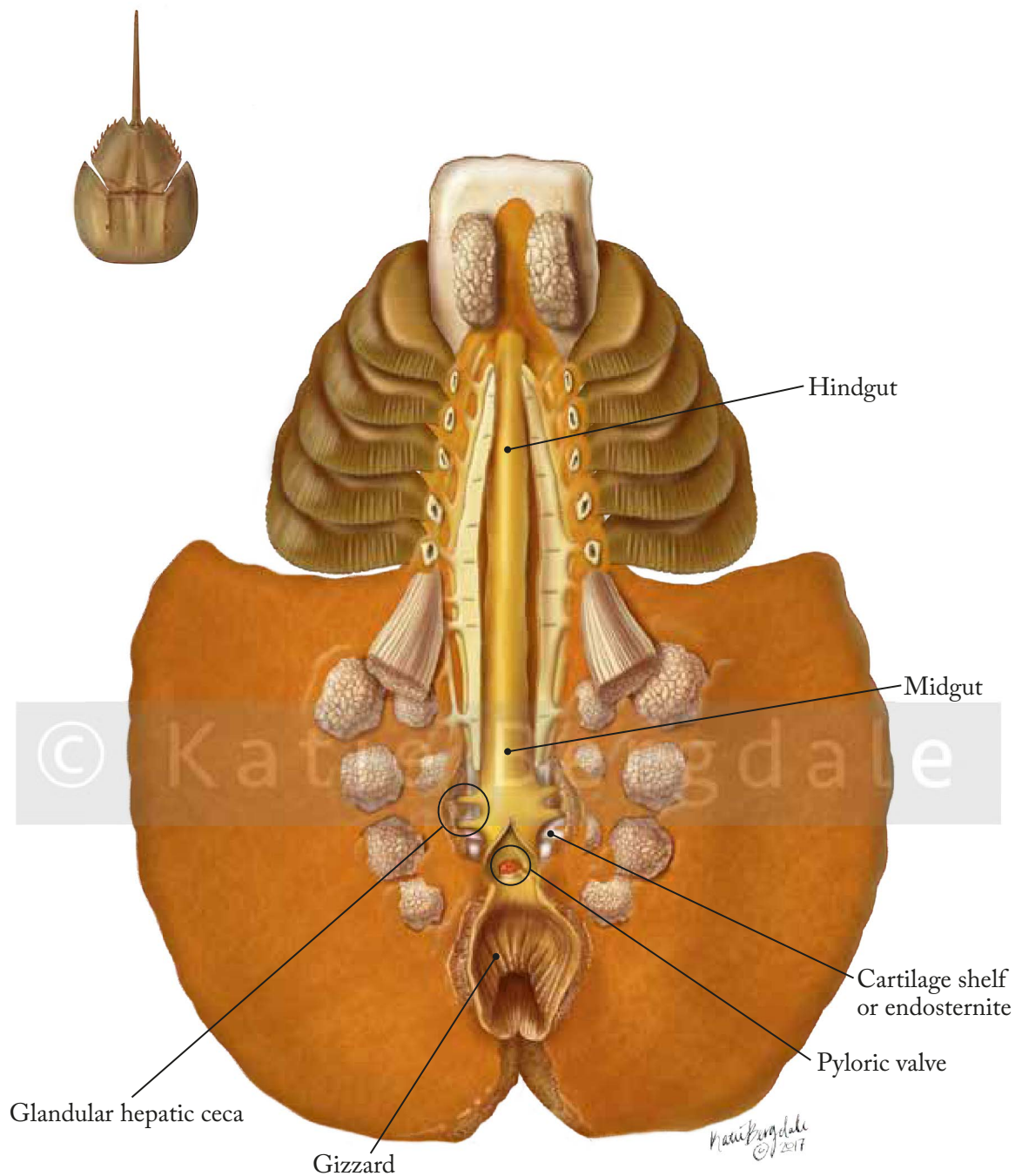


**FIG. 16:** Necropsy by Ventral Approach, Step 12: Turn over the specimen to expose the ventral side. (1) Starting at the opening of the mouth, cut anteriorly through the arterial ring, brain, and (2) the esophagus up to the opening of the gizzard. Gently expose the gizzard from the surrounding tissue to view the external structure.

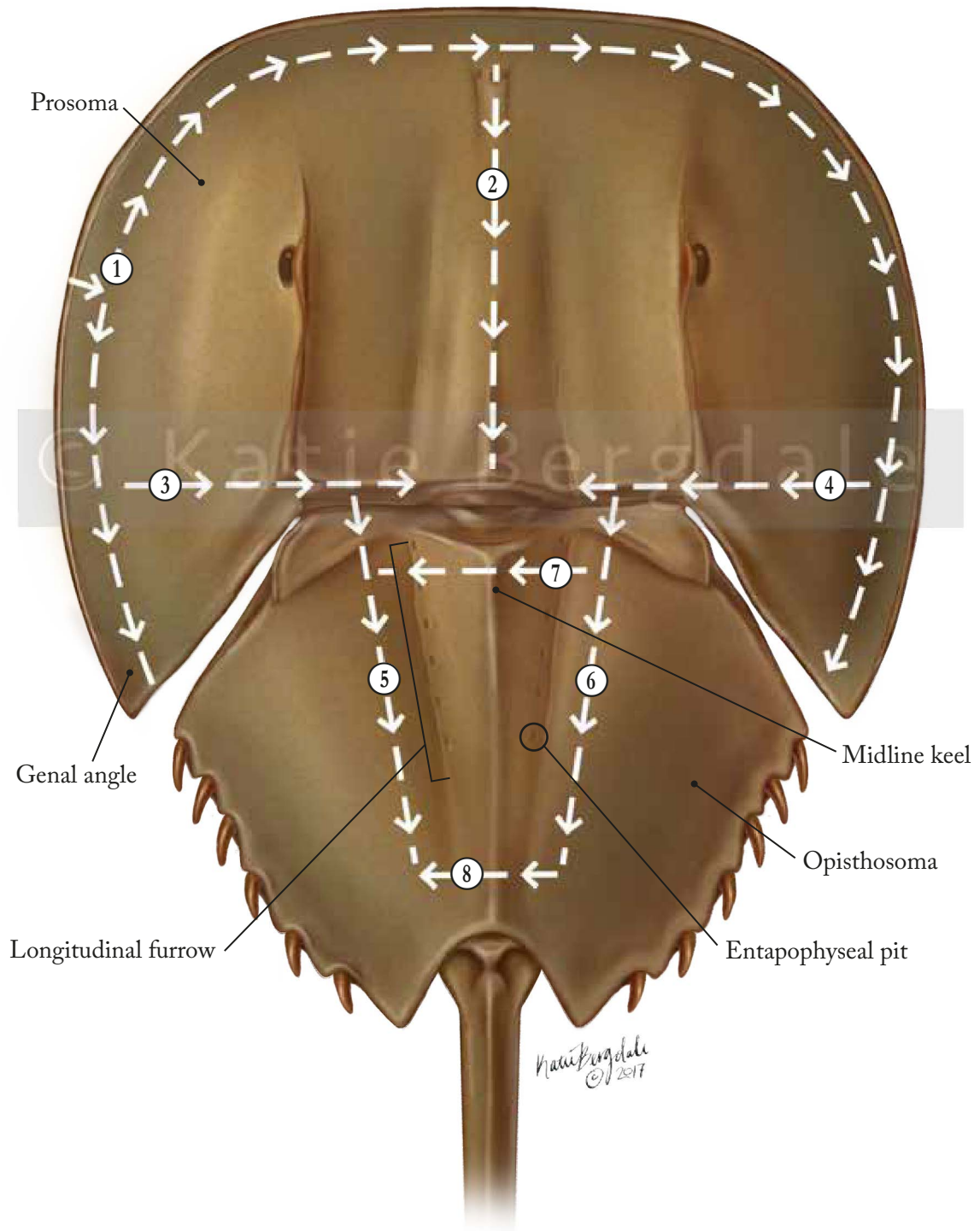




**FIG. 17:** Necropsy by Ventral Approach, Step 13: Turn over the specimen to view the dorsal side again. Cut open the gizzard to expose the inner denticles and the pyloric valve. Carefully divide the heart and pericardial sac at the midline to expose the entire intestine.



**FIG. 18:** Necropsy by Ventral Approach, Step 14: Observe the intestine (midgut and hindgut) and the four glandular hepatic ceca or connections from the midgut to the hepatopancreas.



**FIG. 19:** Necropsy by Dorsal Approach: (1) Make a cut perpendicular to the carapace and continue cutting around the edge of the prosoma, about 1 cm medial. (2) Cut along the midsagittal plane or midline to the arthroal membrane, but not through it. (3) Begin cutting parallel to the arthroal membrane and cut to it. (4) Repeat on other side. Cut the dorsal leg muscle attachments and peel away the prosoma. (5) Cut toward the telson along the longitudinal furrow. (6) Repeat this on opposite side. (7) Cut through the midline keel at the anterior opisthosoma to preserve the arthroal membrane. (8) Cut through midline keel at the posterior end of the opisthosoma to connect cuts 5 and 6. Remove this part of the carapace. Observe the underlying anatomy. (For a detailed description, see *Dorsal Approach*.)

### **Horseshoe Crab Handling and Hemolymph Extraction Technique**

This two-step phlebotomy technique was created by the National Aquarium to preserve hemolymph for biochemical assays (drawn into a sterile syringe) and for total hemocytes counts (drawn directly into anticoagulant) (Fig. 20). This procedure was specifically developed to be minimally invasive to horseshoe crabs and to prevent coagulation during hemolymph extraction for more successful analysis. Three people are needed to perform this hemolymph extraction technique: one to handle and hold the horseshoe crab and the two others to extract hemolymph and handle the two syringes. Proper handling requires the technician to firmly hold onto the prosoma of the horseshoe crab, while gently pushing down the opisthosoma with their thumbs to widen the access to the phlebotomy site at the arthrodial membrane, located between the prosoma and opisthosoma.

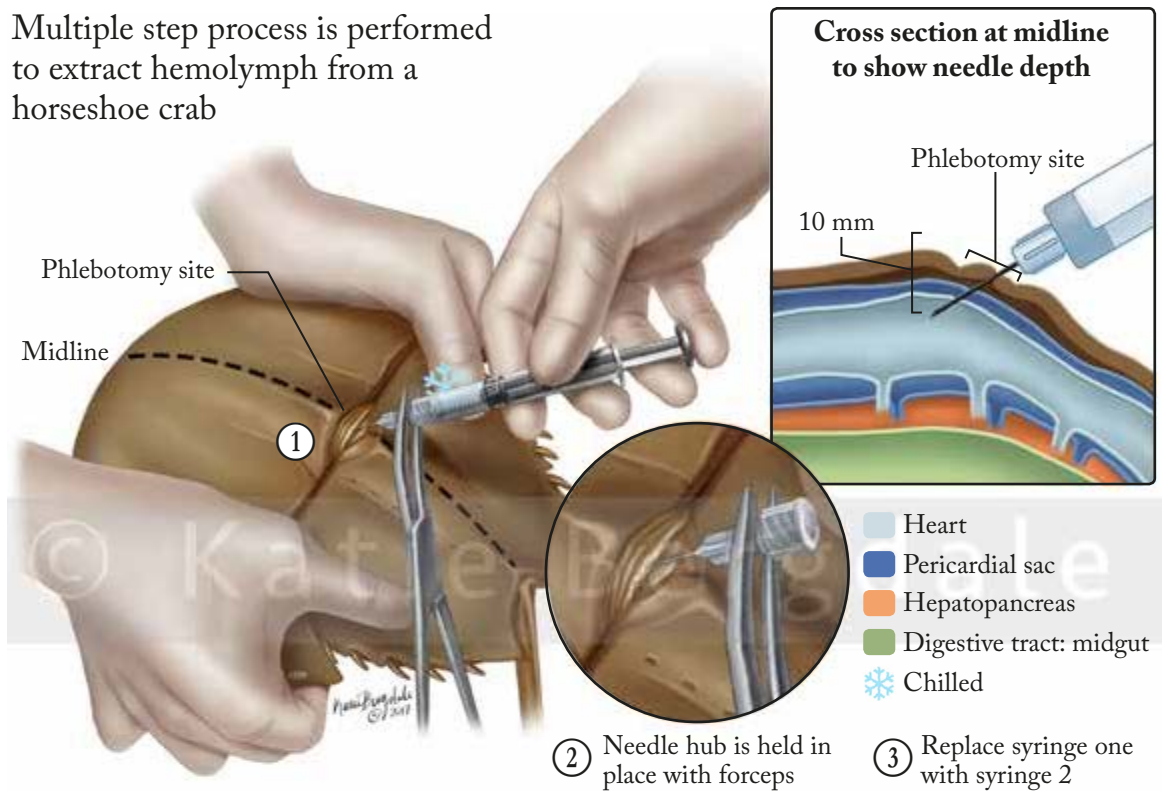
A multi-step process is performed to extract hemolymph from a horseshoe crab. This procedure is performed without withdrawing the needle, which makes the procedure less invasive by minimizing puncture wounds. The first syringe is empty, pre-chilled on ice, and used to remove 1-2 mL of hemolymph. Forceps are used to hold the needle hub in place while detaching the syringe from the needle. The second syringe, also chilled on ice, containing 1.5 mL of cold anticoagulant is quickly attached to the needle hub and 0.5 mL of hemolymph is drawn directly into the cold anticoagulant (1:4 dilution). Hemolymph from the first syringe is placed in a chilled lithium heparin vacuum tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and the contents of the second syringe are transferred to a chilled sterile plain tube. The tube is mixed well, and 200  $\mu$ L of the anticoagulant/hemolymph is transferred into 800  $\mu$ L of chilled 10% neutral buffered formalin (1:5 dilution; final dilution of mixture is 1:20). The heparin tube is centrifuged and filtered (Serum Filter System, Fisher Scientific, Pittsburgh, PA) to prepare a cell-free plasma sample for the biochemical panel. The formalin fixed

cells are further diluted (1:5) in Natt & Herrick diluent (final dilution 1:100) for the hemocytometer count using the standard hematology formula for WBC count (refer to Appendix B for an enlarged version of the bottom section of Fig. 20).

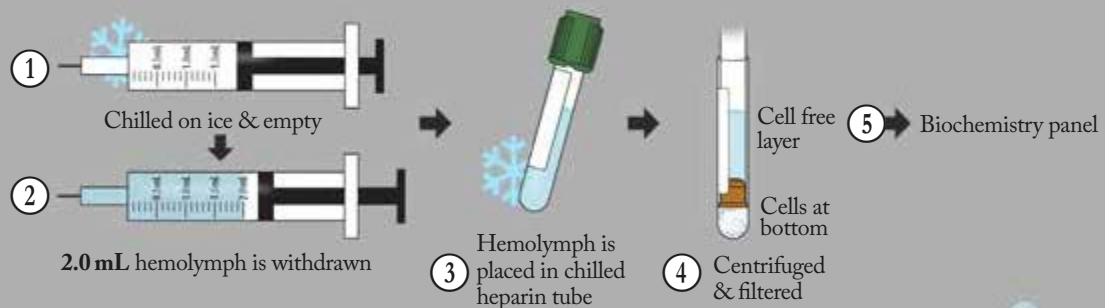
An inset in Figure 20 of a midsagittal view of the horseshoe crab illustrates the proper placement and depth of the needle at the center of the phlebotomy site and positioned 10 mm deep to prevent puncture through the pericardial sac. The overall goal of this figure is to teach veterinarians and researchers to perform this minimally invasive extraction procedure developed by the National Aquarium, which reduces puncture wounds and hemolymph coagulation.



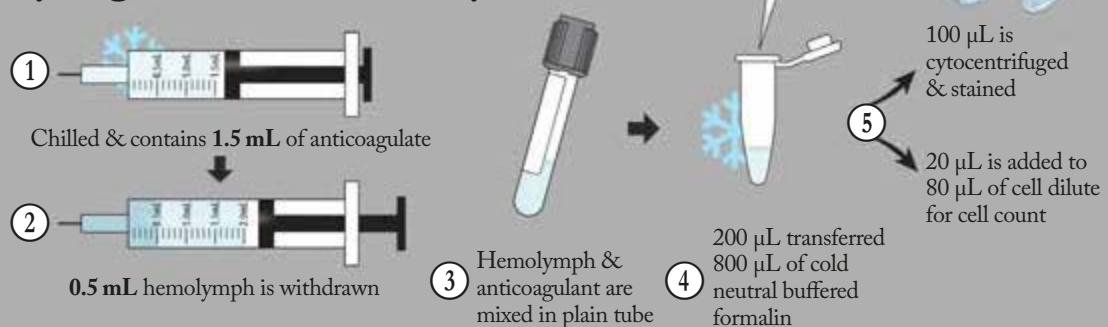
Multiple step process is performed to extract hemolymph from a horseshoe crab



### Syringe 1: Used for biochemistry panel



### Syringe 2: Used for hemocyte counts



**FIG. 20:** Horseshoe Crab Handling and Hemolymph Extraction Technique: The top section shows how to draw a hemolymph sample. The bottom section shows the sample handling and processing.

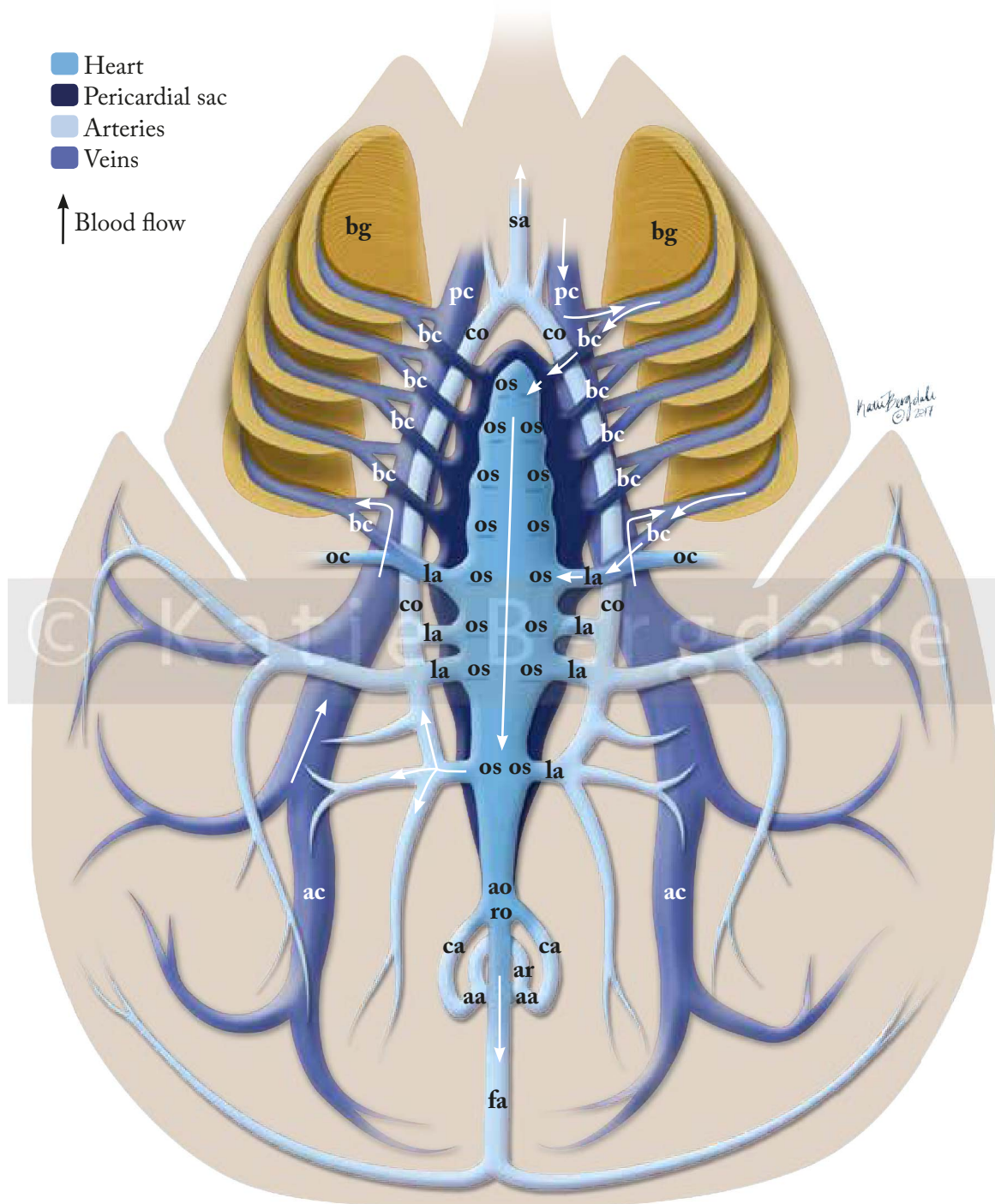
### Circulatory System

The dorsal tubular heart is encased within the pericardium but shown diagrammatically for clarification of the borders of each structure (Fig. 21). The following blood pathway description is from the dissection notes, *Anatomy and Diseases of Limulus Dissection Instructions* provided by Dr. Roxanna Smolowitz. It includes additional text for clarification.

Blood is pumped out through three large anterior arteries [a pair of cephalic arteries that become the aortic arches that join the arterial ring and the frontal artery that extends to the anterior prosoma and then branches laterally] and four pairs of lateral arteries [that] open from the heart at points opposite the first four pair of ostia and led into a pair of collateral arteries, one located on each side of the heart. [These are positioned from the first pair of ostia to the last pair of book gills and merge together anterior to the anus creating the superior abdominal artery.] These arteries terminate in tissue sinuses. The blood collects ventrally into two large longitudinal sinuses [labeled posterior cardinal sinuses ventrally and anterior cardinal sinuses anteriorly]. From the ventral sinuses [or posterior cardinal sinuses] the blood flows into five book gills, through five branchio-cardiac canals, where it is oxygenated. The exchange of gases takes place between the blood in the lamellae and the surrounding water. The gill movement circulates the water over the lamellae and pumps blood in and out of the lamellae. During the forward movement, the lamellae become filled with blood. Backward movement expels the blood and returns it to the pericardium (5).

The blood collects anteriorly in the anterior cardinal sinuses and flows to the book gills. Understated, realistic instructional color is used to distinguish between the arterial

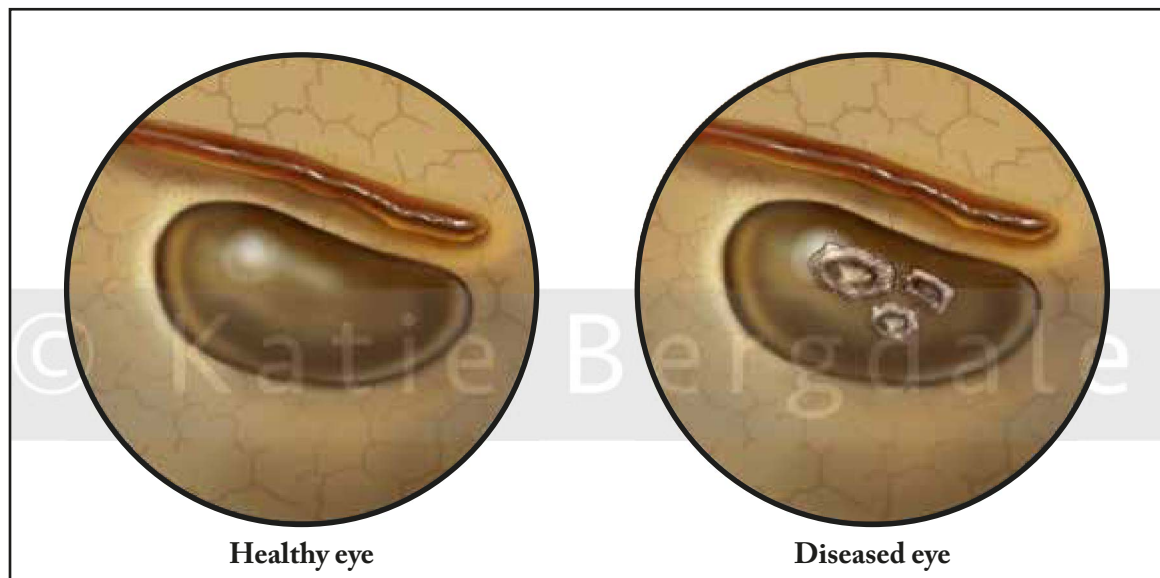
and venous supply. The colors of the heart and pericardial sac remain consistent with previous illustrations showing these structures. Horseshoe crab hemolymph is blue due to the presence of copper in the oxygen-carrying protein, hemocyanin. Arrows are used to represent the pathway of blood throughout the figure.



**FIG. 21:** Circulatory System: **aa** aortic arch; **ac** anterior cardinal artery; **ao** aortic valve; **ar** arterial ring; **bc** branchio-cardiac canals; **ca** cephalic artery; **co** collateral artery; **fa** frontal artery; **la** lateral artery; **os** ostia; **bg** book gill; **sa** superior abdominal artery; **pc** posterior cardinal vein; **oc** opercular canal; **ro** rudimentary ostium

### Common Lesions in Horseshoe Crabs

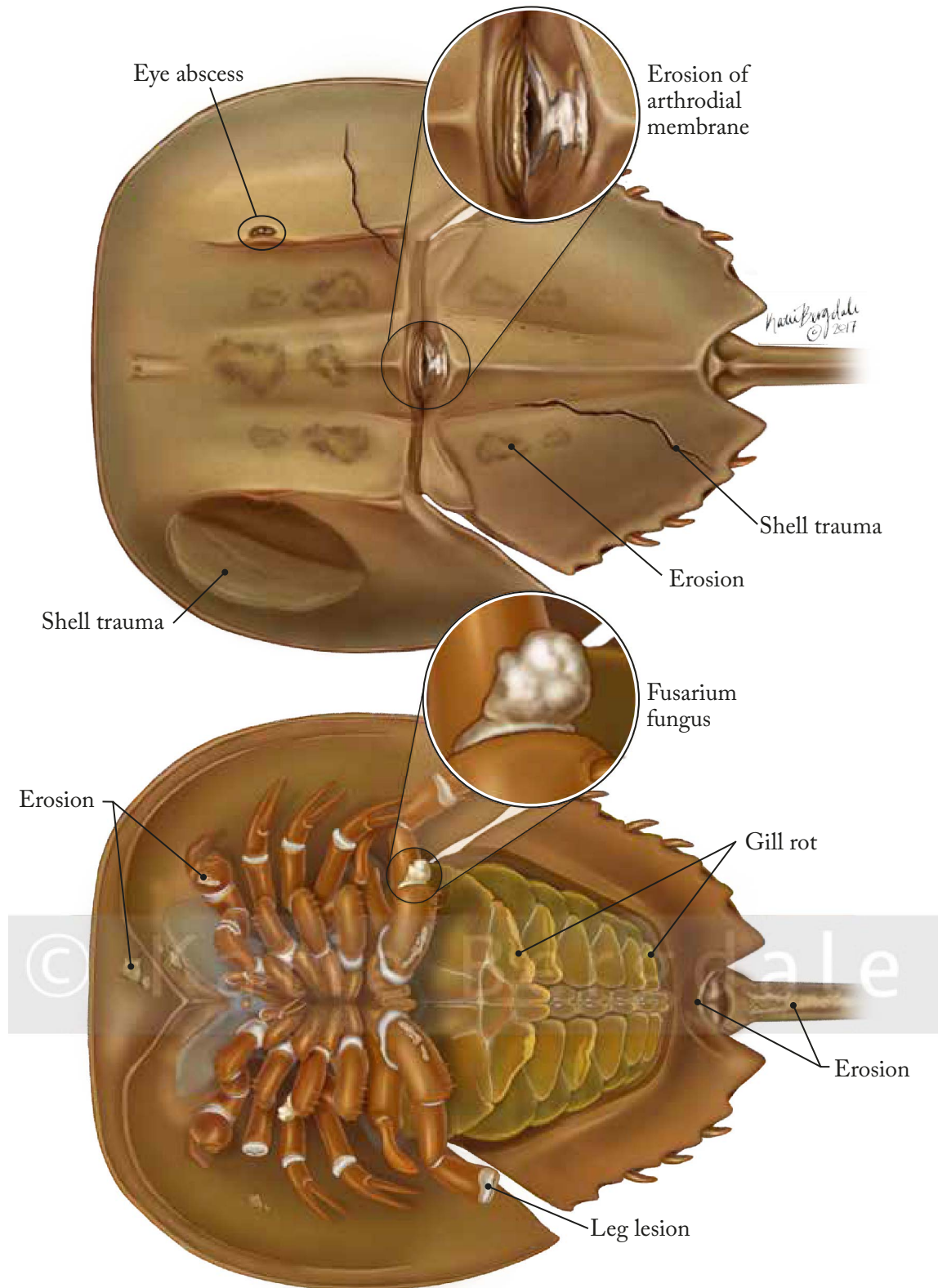
Horseshoe crabs are susceptible to many bacterial diseases which can cause shell erosion and/or trauma-based lesions. Their eyes can erode and form abscesses. A comparison of a diseased eye to a normal eye is shown in Figure 22.



**FIG. 22:** Eye Comparison: A diseased eye has abscesses and erosion present compared to a healthy eye.

Large, discolored, irregular patches of erosion on the surface of the carapace, commonly known as shell disease, is common in both free-ranging horseshoe crabs and those under managed care. Other lesions can occur from physical trauma such as a crushed carapace or fracture (Nolan and Smith 2009) (Fig. 23). Additionally, smaller patches of erosion on the legs, telson, and arthrodial membrane can occur from a variety of infectious agents such as algae, fungi, bacteria, and parasites (Smith 2012). As an individual ages, it is more susceptible to disease. Erosion and necrosis of the book gills are directly related to a bacterial disease called gill rot. This causes actual decay of the book gills, yellowing of the gill opercula, and blackening of the individual lamellae. Shell trauma and lesions to the appendages (legs, gills, or telson) can occur due to improper handling, predators, or environmental factors. The fungus, *Fusarium*, can be seen as fast growing, large white globular lumps around the segments of the legs.

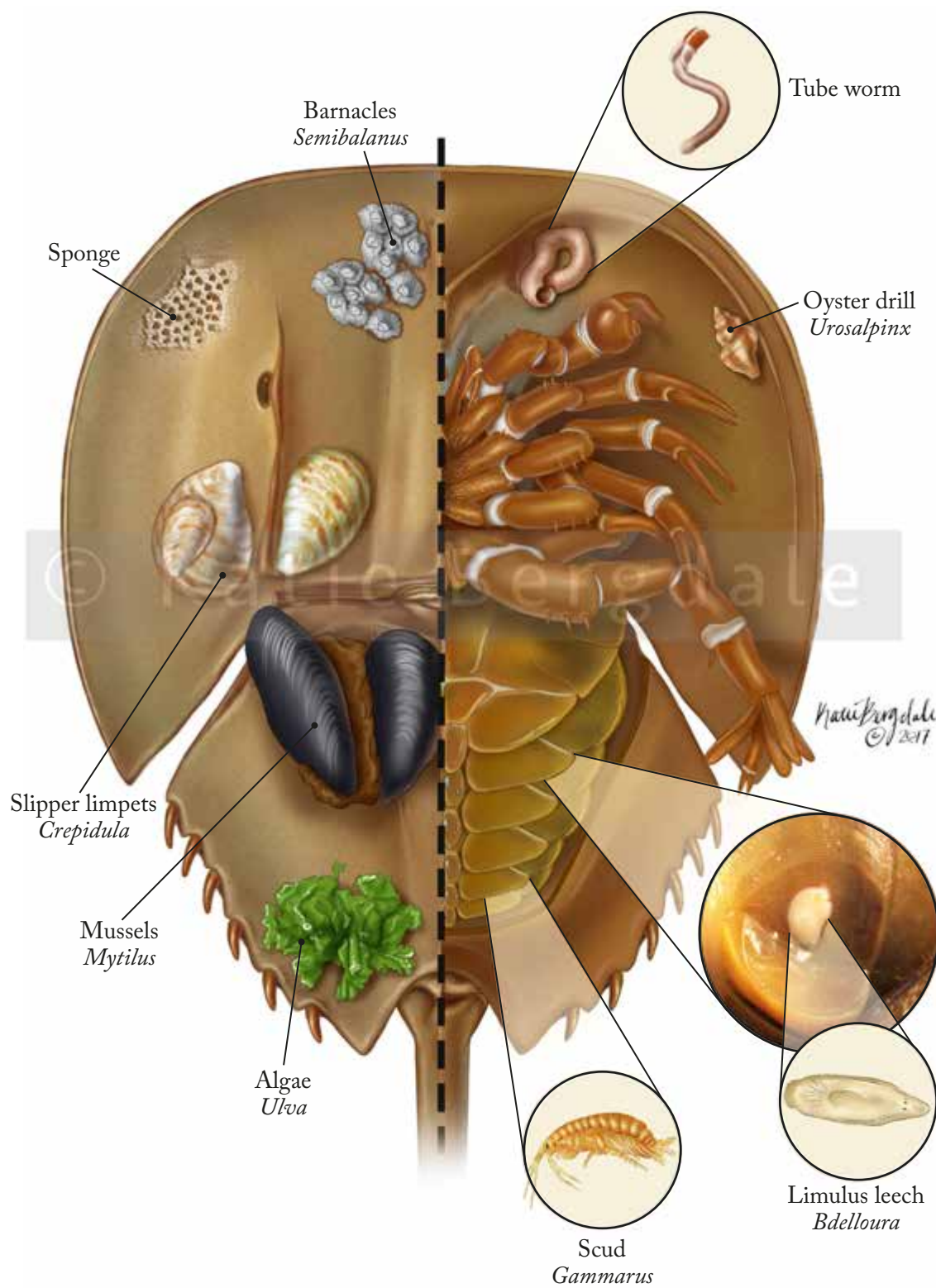




**FIG. 23:** Common Lesions in Horseshoe Crabs: These occur on the carapace, arthroal membrane, appendages, eyes and telson.

### **Inhabiting Organisms**

Many sessile organisms inhabit the carapace or book gills of the horseshoe crab (Fig. 24). This interaction is known as epibiosis, a nonsymbiotic relationship between a basibiont, a host (the horseshoe crab), and an epibiont (a sessile organism)(Shuster et al. 2003). The most commonly observed species that have this interaction with the horseshoe crab in this matter include: the common sponge, mussels (*Mytilus*), barnacles (*Semibalanus*), scuds (*Gammarus*), oyster drill (*Urosalpinx*), Limulus leech (*Bdelloura*), Slipper limpets (*Crepidula*), algae (*Ulva*), and tube worms.



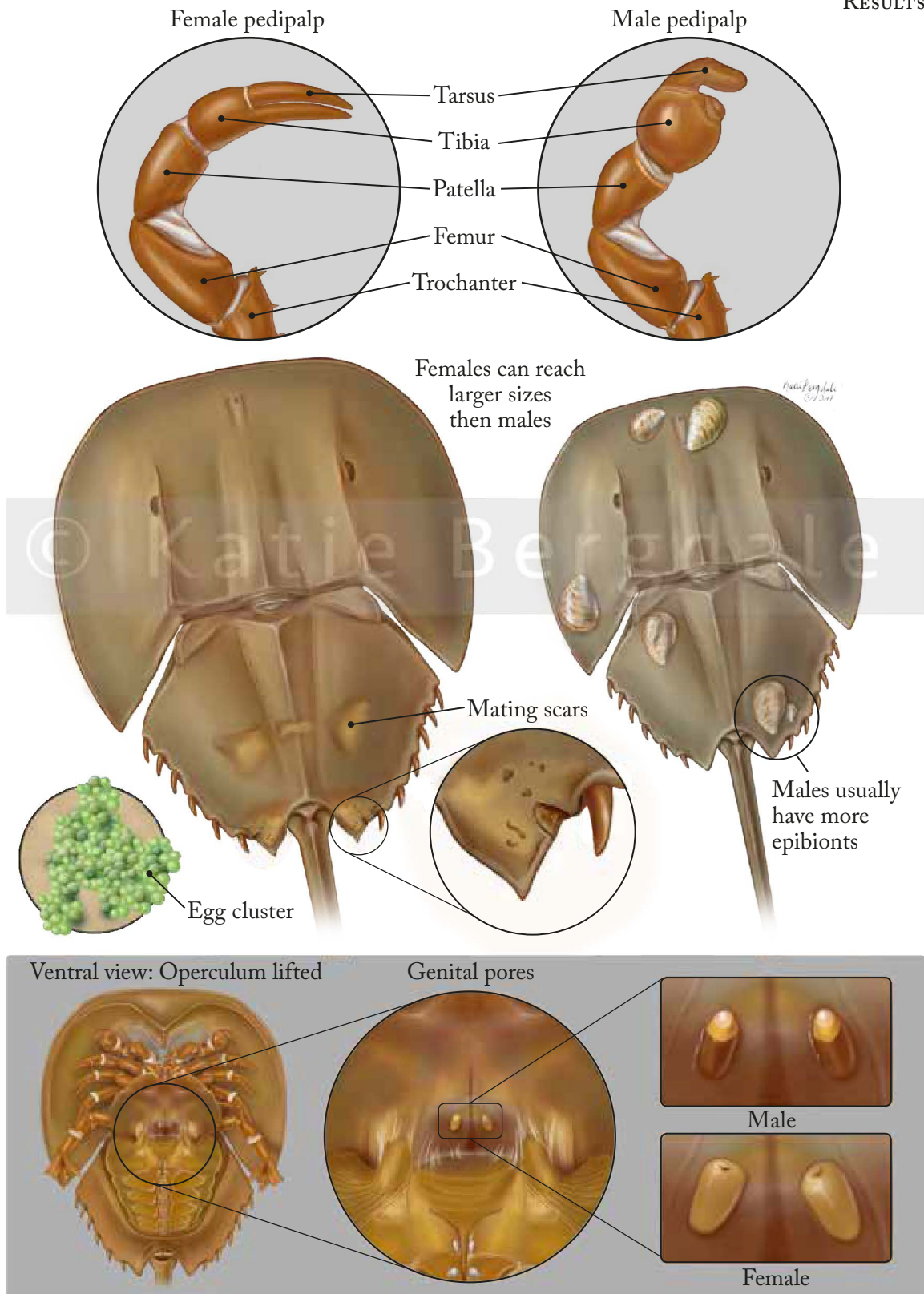
**FIG. 24:** Inhabiting Organisms: Commonly observed sessile organisms include: common sponge; mussels: *Mytilus*; barnacles: *Semibalanus*; scud: *Gammarus*; oyster drill: *Urosalpinx*; limulus leech: *Bdelloura*; Slipper limpets: *Crepidula*; algae: *Ulva*; tube worms.



### Sex Comparison

Briefly, the differences between males and females are as follows: the shape of the pedipalp claw, the overall body size of each sex, the shape of the paired genital pores, and unique characteristics seen on the outer carapace (Fig. 25). The male's pedipalp has a specialized large bulbous claw, or the tarsus, and tibia, used to firmly hold the female's opisthosoma during mating. The females' pedipalp is ambulatory leg. The other three segments of the pedipalp, the patella, femur, and trochanter, are similar in both sexes (detailed illustration of pedipalps, Fig. 27). Adult females are 18-19 inches in prosomal length while adult males are 14-15 inches in length. Each sex has two genital pores or gonophore openings which secrete either eggs or sperm during mating. The pores are positioned on the ventral surface of the genital operculum. Genital pores vary in shape and color between sexes. The male's gonophores are elevated and tubular in shape with bright orange tips; the female's are slit-like, flattened and less saturated in color.

There is a difference in the appearance of the carapace between sexes. Females' opisthosomas consistently have mating scars in a butterfly pattern seen on the dorsal surface caused by the male's prosoma rubbing against it during mating. The other noticeable scarring is an indent caused by a male's pedipalp claw, specifically the tarsus, firmly holding onto the most posterior triangular point of the female's opisthosoma. In contrast, a male's carapace is usually more heavily covered by epibionts or sessile organisms. There are two possible explanations: first, males stop molting their shells once they reach sexual maturity whereas females continue to molt (Carmichael et al., 2003); second, both sexes experience a terminal molt but males reach sexual maturity much earlier than females (Smith, Mandt, and MacDonald, 2009). However, it has been observed at the MBL that when housed in managed care, adult horseshoe crabs stop molting after reaching sexual maturity (pers. comm. Amy Hancock-Ronemus). An inset on Figure 25, positioned to the left of the female, illustrates the egg clusters they lay.



**FIG. 25:** Sex Comparison: The main difference between males and females is the pedipalps. The male has a specialized pedipalp designed to firmly hold onto a female's opisthosoma. Sexes differ in size. Females average in 18-19 inches (prosoma width) and males are approximately 14-15 inches (prosoma width).

### Cross Section at Midline

A simplified illustration of a cross section of the horseshoe crab helps explain the position of the anatomical systems in relation to one another (Fig. 26). These systems include digestive, excretory, circulatory, and nervous. A cross section shows the pathway of food through the digestive system. Food is ground by the gnathobase and pushed through the mouth, aided by the chelicerae. Food then travels through the sclerotized esophagus which contains longitudinal folds that extend to the dilated crop. The gizzard, or grinding chamber, immediately follows, and contains denticles on longitudinal circular folds that further grind up the food. Any large indigestible particles are regurgitated via the esophagus. The digestible food continues through the pyloric valve and into the intestine, specifically the nonsclerotized midgut. Two pairs of ducts called glandular hepatic ceca, ramify throughout the hepatopancreas. Food is passes through the hindgut, and out through the sclerotized rectum and anus.

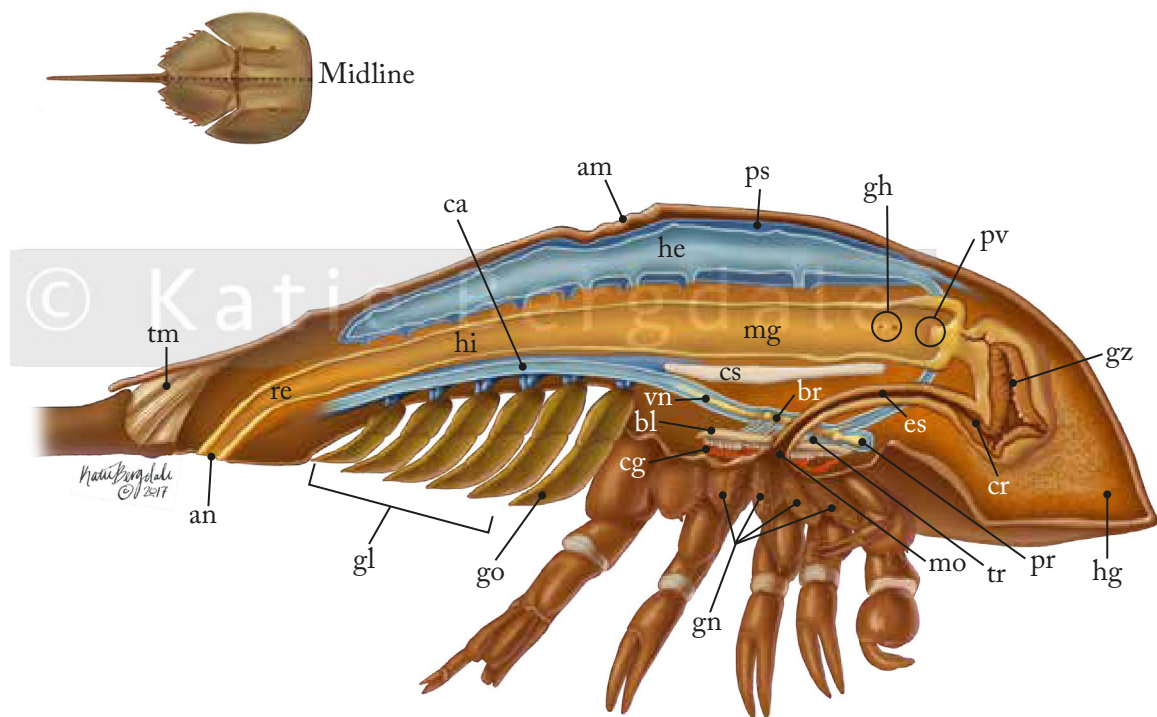
Horseshoe crabs have a simplified excretory system compared to other invertebrates. The excretory system consists of four pairs of bright reddish orange glands, referred to as the coxal glands due to their position within musculature of the coxa leg segments (Shuster, et. al., 2003). Waste first collects in the coxal glands and transferred into a common chamber then moved into the convoluted tubules to the paired elongated bladders. Waste is finally excreted through a small duct termed the excretory pore, or nephropore on the posterior surface of the fifth appendage, or last walking leg (Smolowitz, 1999). For a detailed view of the excretory system see Figure 11.

The nervous system is unique because it is almost entirely enclosed within the arterial system. The brain is positioned within the arterial ring, forming a collar around the esophagus. Anteriorly, the brain forms a bulge called the protocerebrum. “The lateral [region] is a fusion of the tritocerebrum and the ganglia for all the remaining first seven segments” (Smolowitz, 1999). The brain gives off a thick posterior branch called the

ventral nerve cord that continues posteriorly, within the abdominal artery, to the telson.

The ventral nerve cord has five ganglia and lateral nerves that supply the opisthosoma and opisthosomal appendages (Smolowitz, 1999).

The heart is completely encased within the pericardial sac. For a detailed description of the entire circulatory system, see Figure 21.

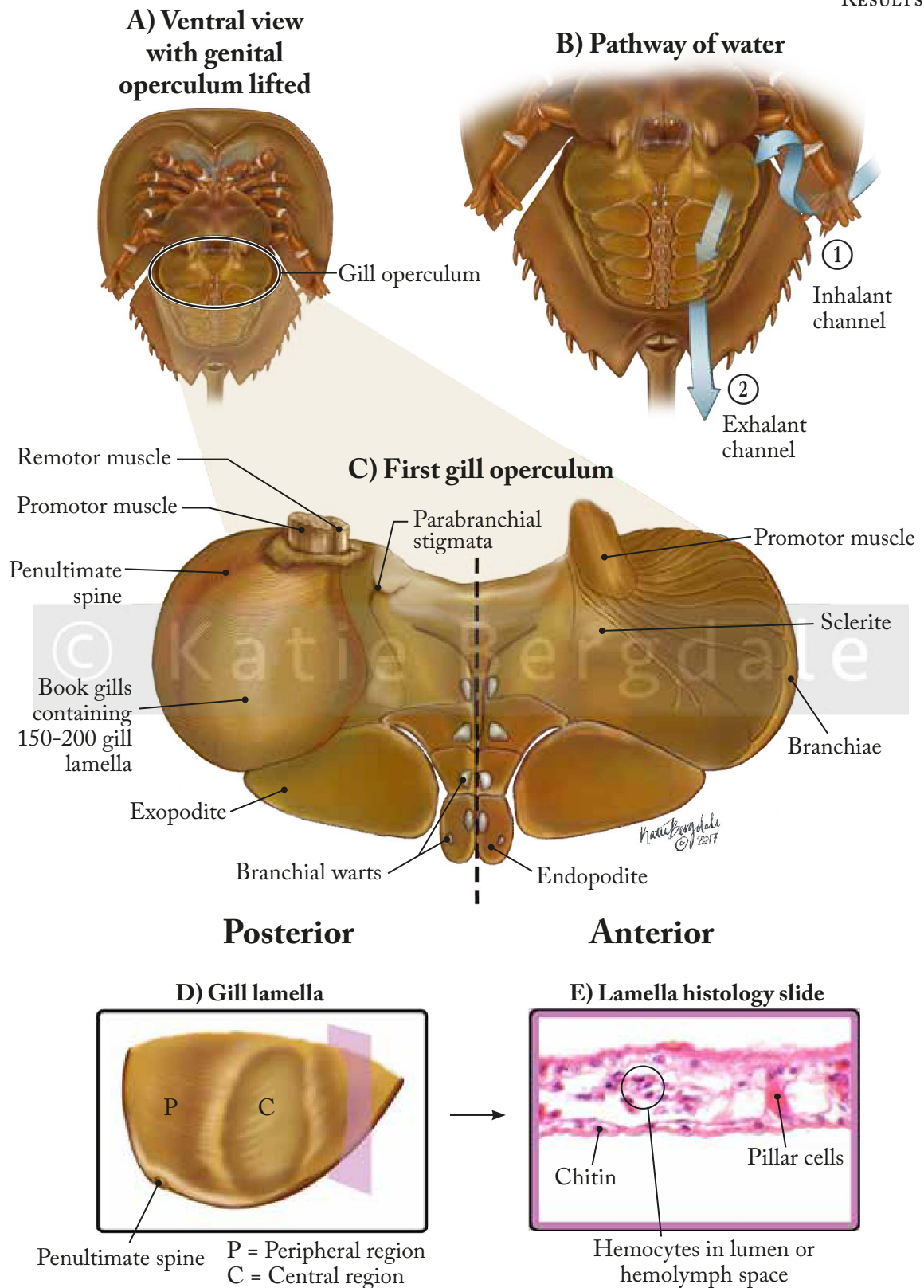


**FIG. 26:** Cross Section at Midline: **am**: arthroal membrane; **an**: anus; **bl**: bladder; **br**: brain within arterial ring; **ca**: coelomic artery (abdominal artery); **cg**: coxal gland; **cr**: crop; **cs**: cartilage shelf or endosternite; **es**: esophagus; **gh**: glandular hepatic caeca; **gl**: gill opercula; **gn**: gnathobase; **go**: genital operculum; **gz**: gizzard; **he**: heart; **hg**: hepatopancreas and gonadal tissue; **hi**: hindgut; **mg**: midgut; **mo**: mouth; **pr**: protocerebrum; **ps**: pericardial sac; **pv**: pyloric valve; **re**: rectum; **tm**: telson muscles; **tr**: tritocerebrum; **vn**: ventral nerve cord See full page illustration in Appendix A.

### Gill Anatomy

The horseshoe crab has specialized appendages for respiration known as book gills. Figure 27 illustrates a detailed view of the anterior and posterior surface of the first gill operculum (C), a single gill lamella (D), and a histological image of multiple gill lamellae (E). It also shows the pathway of water between the prosoma and opisthosoma at the inhalant and exhalant channels (B). Water is drawn through these channels by pulsations made by the gill opercula (Shuster, et. al., 2003). Water enters the inhalant channel, flows across the book gills and exits at the exhalant channel (Fox, 2007). Horseshoe crabs possess five gill opercula, each containing five paired laterally positioned book gills. Each book gill comprises on average 150 to 200 lamellae or leaflets. In an average adult this amounts to 11,000 cm<sup>2</sup> of surface area across in which respiration occurs (Shuster, et. al., 2003). Horseshoe crabs move their book gills in a wavelike movement by lifting and lowering each gill opercula in a continuous motion, actively irrigating their lamellae with seawater (Shuster, et. al., 2003). Two muscles control this wavelike movement: the promotor and remotor muscle. A single gill lamella contains two regions: the peripheral or outer and the darker, pigmented central region. Shown histologically, each lamella consists of two epithelial cell layers on the outer edges, which enclose a lumen (or hemolymph space) containing hemocytes that are held apart by pillar cells allowing efficient gas exchange (Smolowitz, 1999).

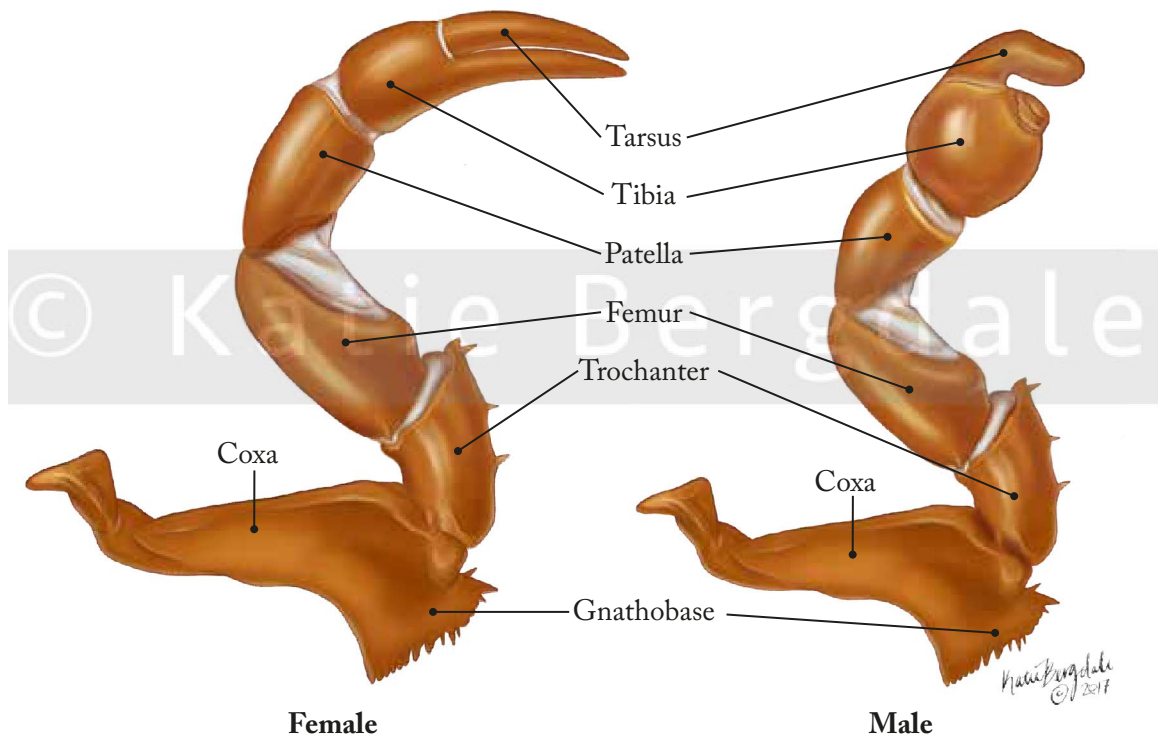




**FIG. 27:** Gill Anatomy: A) Ventral view of a horseshoe crab with the genital operculum lifted; B) Pathway of water between prosoma and opisthosoma at inhalant and exhalant channels; C) Posterior and anterior view of the first gill operculum; D) Single gill lamella; E) Histology slide of a single gill lamella

### Leg External Anatomy

The anatomy of the legs consists of the similar morphology and nomenclature to vertebrates. As described previously, the pedipalp claws are specific to each sex. However the anatomy is the same between sexes proximal to the claw comprising of the following structures: patella, femur, trochanter, and the coxa which contains a part of the gnathobase (Fig. 28). The ambulatory legs have a long pincher claw and consist of a tarsus, tibia, patella, patella groove, femur, trochanter, and the coxa containing a part of the gnathobase and endite (Fig. 29). The swimmer leg consists of several segments distally housing four curved blades and a small claw comprising the pretarsus and tarsus. The leg segments continue proximally as the tibia, patella houses a spur, femur, trochanter, and coxa that contains a flabellum, epipodite suture, and a part of the gnathobase (Fig. 30).



**FIG. 28:** Female and Male Pedipalps: The second prosomal appendage

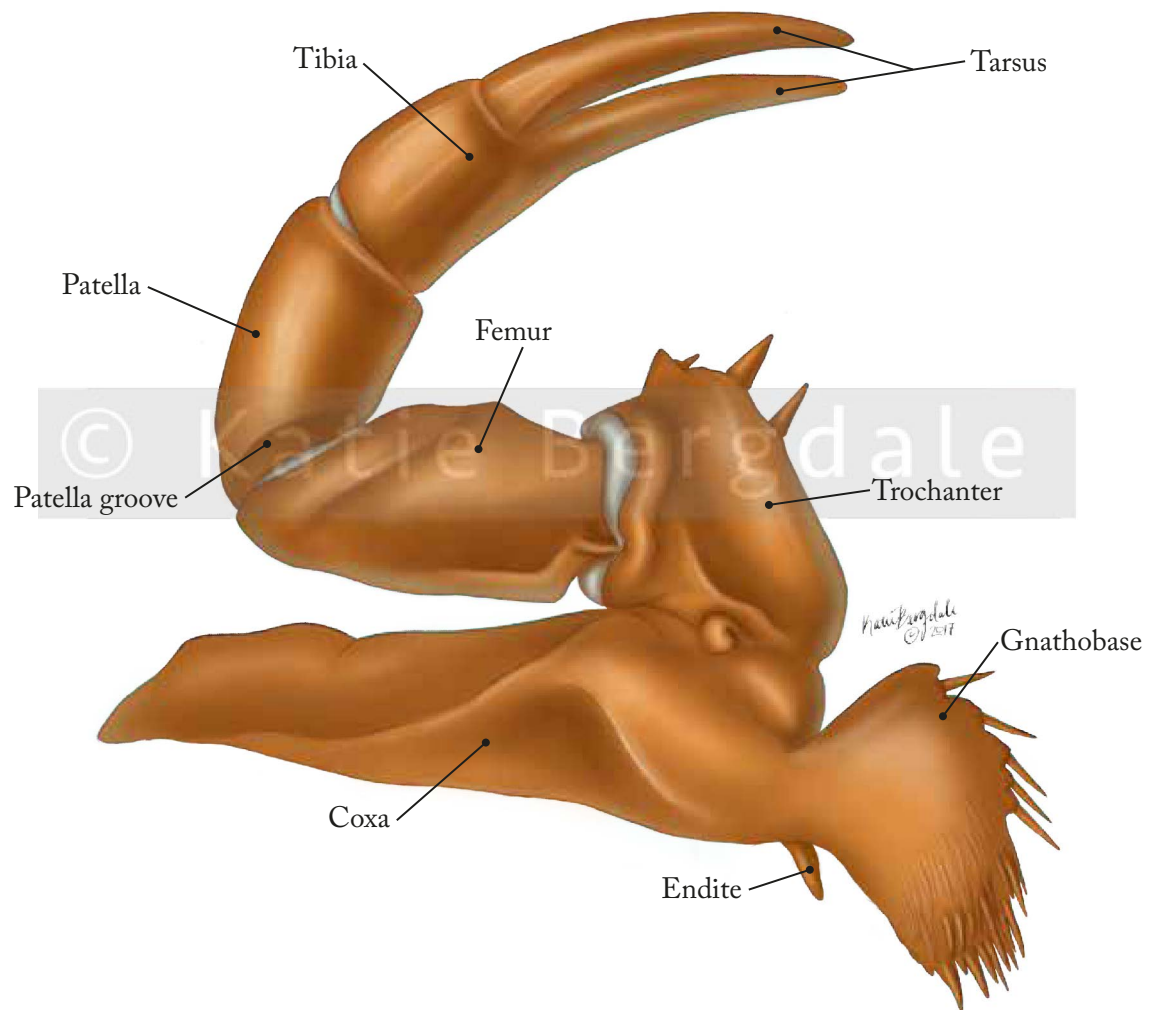


FIG. 29: Walking leg: The third through fifth prosomal appendages



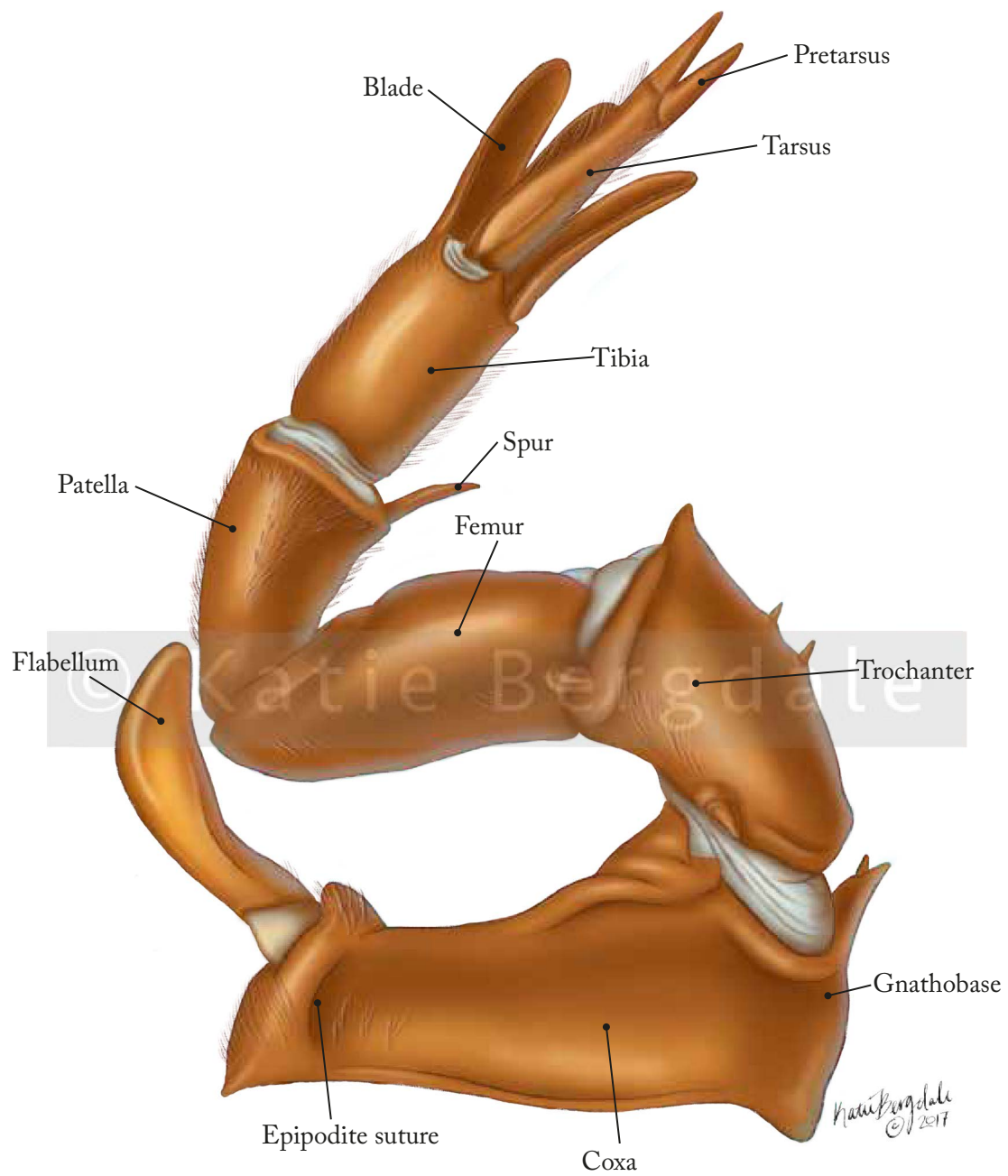


FIG. 30: Swimmer leg: The sixth prosomal appendage

### **Asset Referral Information**

The illustrations resulting from the work of this thesis will be partially found at **www.katiebergdale.com**. Access to the illustrations can be granted by contacting the author at **katiebergdale@gmail.com** or through the website of the Department of Art as Applied to Medicine at Johns Hopkins University School of Medicine: **<http://medicalart.johnshopkins.edu/>**.

The National Aquarium in Baltimore, MD will retain copies of the illustrations for intended future publications. The intended journals include: the *American Journal of Veterinary Medicine*, and the *Journal of Exotic Pet Medicine*.

## DISCUSSION

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This study provides multiple illustrations designed to educate veterinarians, aquarists, technicians, and biologists on the proper method to assess the health of a horseshoe crab. In an extensive literature review, no other references were found that provide the same degree of accuracy and detail provided by these illustrations. This project is innovative because the biomedical illustrator assumed a scientific role to directly perform the necropsies and incorporated feedback from multiple institutions to create novel, comprehensive illustrations, which are accurate and well designed for the target audience of veterinarians and researchers. This work can serve as a template for future guides, providing insight for illustrators on effective organization and illustration techniques for this target audience.

### Publication and Production

The illustrations created for the hemolymph sampling technique are intended to be published by the National Aquarium as part of a hemolymph study in the *American Journal of Veterinary Medicine* or similar peer-review journal. The necropsy guide and illustrations are intended to be published in the *Journal of Exotic Pet Medicine*. The illustrations will also contribute to a future horseshoe crab husbandry guide produced in collaboration with several institutions for the Taxon Advisory Group under the Association of Zoos and Aquariums (AZA). The AZA requires the highest standards for accreditation of zoos and aquariums, and publications by the AZA become standard care for a species or taxonomic group. The husbandry guide is a 5-year project, and the hemolymph images would be a contributing component as well. The illustrations and the necropsy guide will be used within the National Aquarium to educate their employees.

Each illustration was designed as a stand alone piece allowing each to be understood by the target audience without other images present (except for the necropsy series that will always appear as a complete series). This approach was planned during the developmental stage in storyboards through an iterative storyboarding process to ensure

clarity and a well constructed composition. Each illustration was also designed to be clear in black and white in case the article, which included an image, was printed.

### **Necropsy Challenges**

These necropsy guidelines were developed to provide an organized workflow and to create a sense of consistency throughout the series. Many attempts were made to create a structured workflow, and to determine the most effective sequential order of dissection to best preserve anatomy. It was discovered that blunt scissors were more effective than a scalpel during dissection due to the tough slippery carapace causing little control over the scalpel. Each approach has its advantages and disadvantages depending on a specimen's health and the desired outcome of the necropsy. With healthier horseshoe crabs, the carapace is very dense and especially difficult to cut. In this case, the ventral dissection approach is recommended due to the easy starting access point in the soft ventral tissue (Fig. 5). If the specimen has a weakened carapace, due to recent molting or disease, the dorsal approach is recommended. It was observed that the dorsal approach preserved the heart and pericardial sac better than the ventral approach, but this may have been due to a diseased carapace, causing the heart to detach from the inner dorsal ligaments that suspend the heart and pericardial sac from the dorsal carapace.

Extreme care must be taken when performing the dissections because the internal structures are fragile and difficult to discern. Many structures were difficult to completely preserve, such as the excretory system, the pericardial sac and heart, and the circulatory system. For example, when removing the leg muscle attachments from the dorsal carapace, one must keep the scissors close to the inner dorsal carapace to avoid tearing the heart and pericardial sac that lies directly ventral to it. Most structures within the horseshoe crab are achromatic and difficult to differentiate. The extensive literature review completed in the beginning of this project provided the knowledge needed to distinguish through this unclear anatomy during dissections.

No healthy specimens were dissected for this project; each specimen dissected was found either dead or ill before dissection. It is possible that some structures could appear differently when comparing healthy and diseased specimens. This is unlikely due to the comparison of existing references of specimens to ensure accuracy of anatomical structures. As in human anatomy, each specimen has some degree of variation. Illustrations were drawn to generalize these anatomical differences. Anatomy depicted in the illustrations was based primarily on direct observation during dissections. The project timeline did not correspond with the horseshoe crab mating season, preventing any dissection of gravid females. Information and photo references were gathered from content experts and current literature to accurately illustrate the gonadal tissues and eggs seen in Figure 9.

The necropsy illustrations created for this project utilize small icons as additional instructive elements to explain the positioning of the specimen during the dissection. Dashed lines with arrows were used to explain the direction of cuts. Detailed insets provide the viewer with more information on the complex anatomy being dissected. All anatomical features seen in the dissection were labeled throughout the illustration series. All illustrations relating to the blood use realistic color, unlike existing illustrations that use human color conventions (arteries: red, veins: blue). This is confusing and inaccurate since their hemolymph is blue.

Overall, the dissections, literature review, and accompanying instructive elements used through the illustrations allow for the series to clearly explain the steps and realistically and accurately represent the anatomical structures seen in a specimen. Previously created illustrations lack this realism.

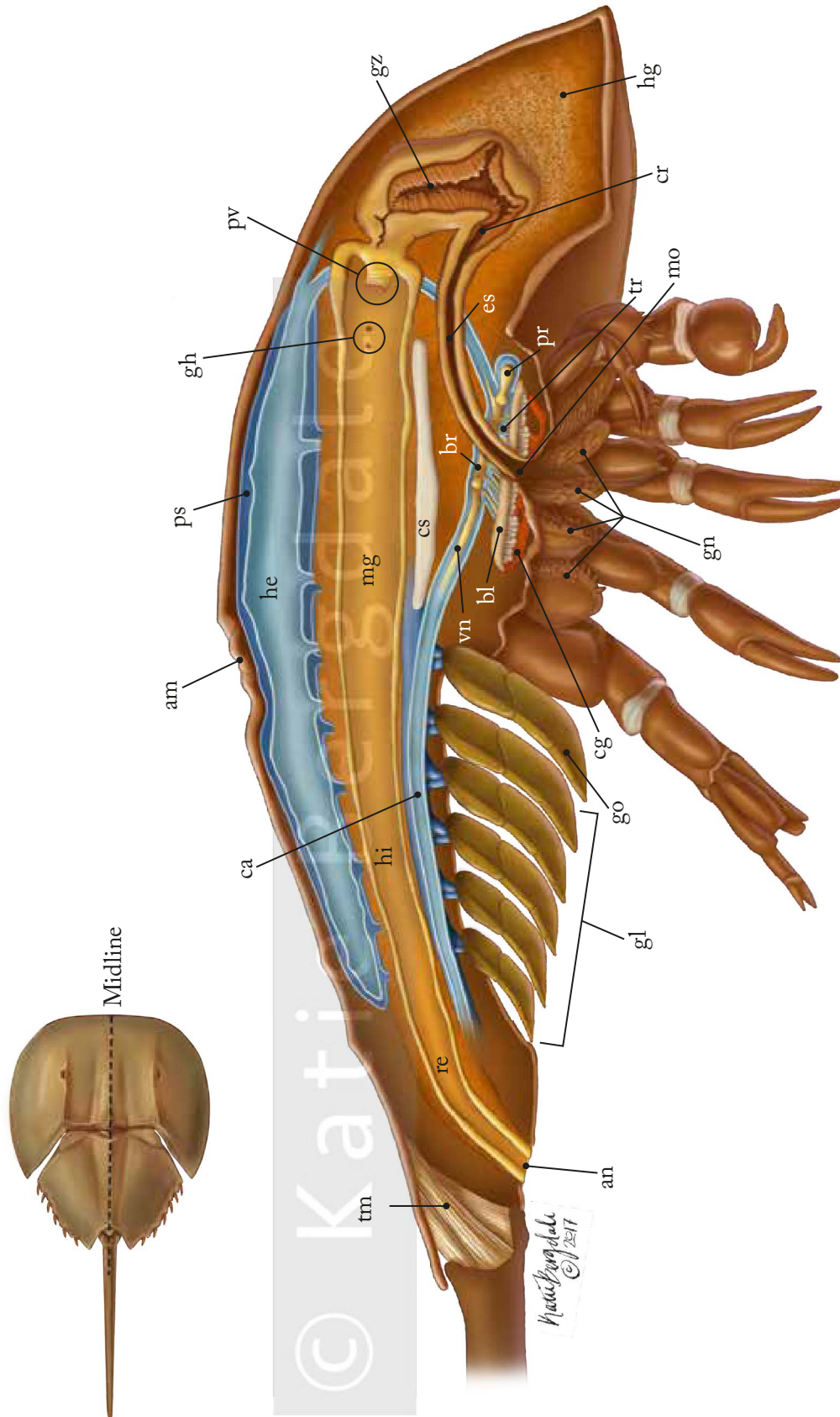
## Conclusion

The main objective of this project was to create multiple illustrations to accompany the specific guidelines created by the National Aquarium to teach the proper health assessment techniques of the horseshoe crab to veterinarians, aquarists, technicians, and biologists. The National Aquarium will use the illustrations and associated text to teach their current employees and students. Plans to publish the material through multiple journals will increase accessibility and awareness.

All illustrations were reviewed by all National Aquarium content experts: Jill Arnold, Brent Whitaker, and Kat Hadfield; by outside aquatic veterinary experts: Roxanna Smolowitz and Amy Hancock-Ronemus, and by the faculty advisor Tim Phelps, to ensure accuracy. This thorough review, critically analyzed by multiple experts, confirms a successful outcome to this project.

Expected benefits of providing didactic illustrations to accompany complex information are improved comprehension and understanding, reduced gaps in knowledge, and future standardized comprehensive material. These illustrations will help to create a better overall understanding of horseshoe crab anatomy and health leading to properly performed health assessments and necropsies. By understanding normal biology of a horseshoe crab, proper diagnoses of an abnormality or disease can occur, contributing to better conservation strategies and proper health maintenance.





**FIG. 31:** Cross Section at Midline: **am**: arthrodial membrane; **an**: anus; **bl**: bladder; **br**: brain within arterial ring; **ca**: coelomic artery (abdominal artery); **cg**: coxal gland; **cr**: crop; **cs**: cartilage shelf or endosternite; **es**: esophagus; **gh**: glandular hepatic ceca; **gl**: gill opercula; **gn**: gnathobase; **go**: genital operculum; **gz**: gizzard; **he**: heart; **hg**: hepatopancreas and gonadal tissue; **hi**: hindgut; **mg**: midgut; **mo**: mouth; **pr**: pericardial sac; **ps**: protocerebrum; **pv**: pyloric valve; **re**: rectum; **tm**: telson muscles; **tr**: tritocerebrum; **vn**: ventral nerve cord

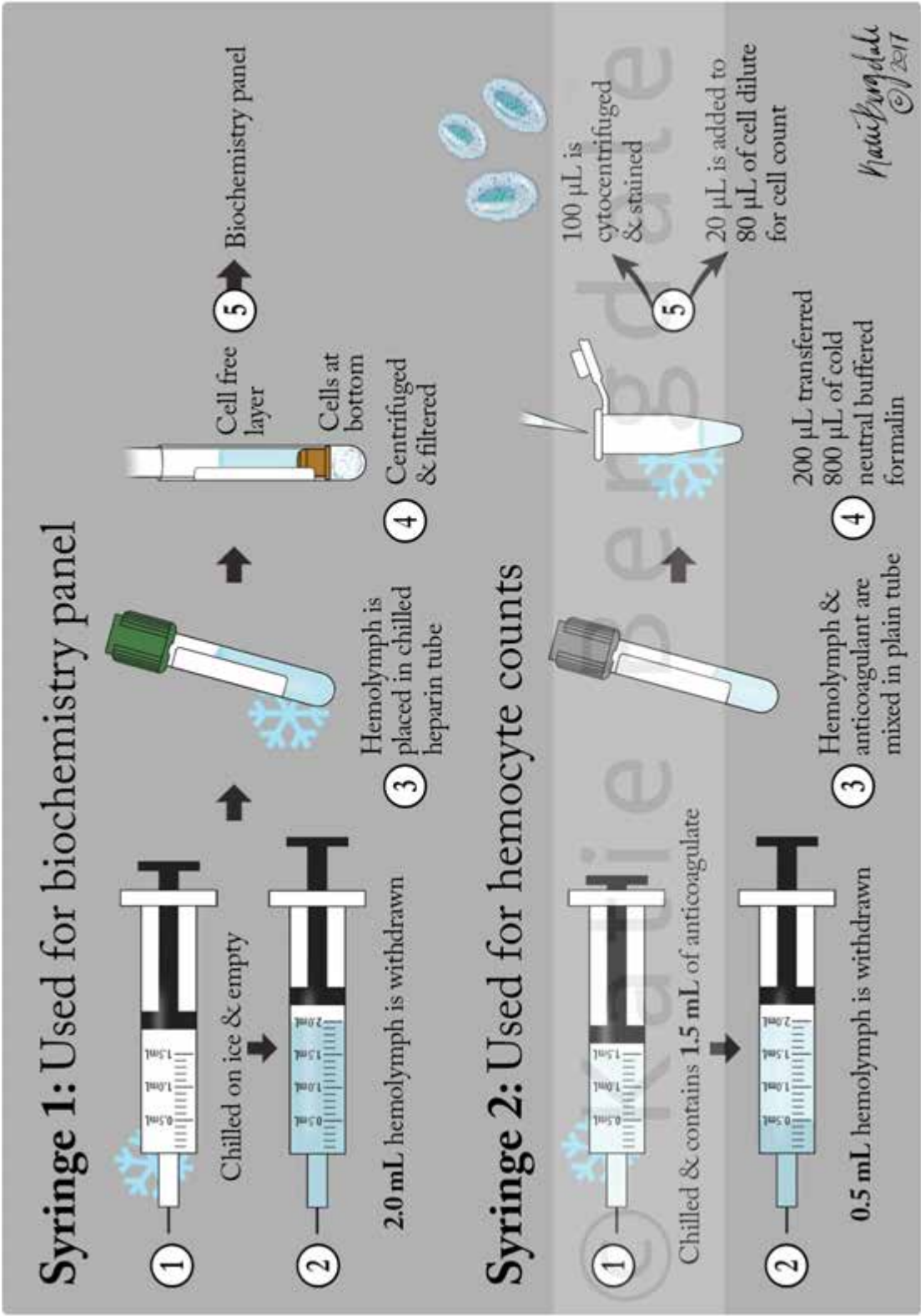


FIG. 32: Horseshoe Crab Handling and Hemolymph Extraction Technique: The bottom section enlarged

### Illustration Outline

<p><b>Clinical techniques:</b></p> <ul style="list-style-type: none"> <li>• Extraction of hemolymph             <ul style="list-style-type: none"> <li>◦ How to handle, hold correctly</li> <li>◦ Procedure methods (specific syringe methods)</li> <li>◦ Phlebotomy site: depth &amp; position</li> </ul> </li> <li>• Tagging             <ul style="list-style-type: none"> <li>◦ Location of tags &amp; how to correctly tag</li> </ul> </li> <li>• Diseases             <ul style="list-style-type: none"> <li>◦ Lesions/Shell trauma</li> <li>◦ Fusarium fungus</li> <li>◦ Diseased eye with abscess vs. to normal</li> </ul> </li> </ul>	<p><b>Illustrations:</b></p> <ol style="list-style-type: none"> <li>1. Crab being handled correctly, showing syringe at hinge point</li> <li>2. Inset showing cross section through crab to explain depth of needle</li> <li>3. Locations of tags on crab (photograph)</li> <li>4. Dorsal image of shell trauma</li> <li>5. Ventral image showing masses and erosions</li> <li>6. Inset comparing a healthy eye to a diseased one</li> </ol>
<p><b>Species that live on/in the crabs</b></p> <ul style="list-style-type: none"> <li>• Limulus leech (<i>Bdelloura</i> spp.)</li> <li>• Barnacles, e.g., <i>Balanus</i> and <i>Semibalanus</i> spp.</li> <li>• Mussels, e.g., <i>Mytilus</i> or <i>Geukensia</i> spp</li> <li>• Slipper limpets (<i>Crepidula</i> spp.)</li> <li>• Oyster drill (<i>Urosalpinx</i> spp.)</li> <li>• Tube worm</li> <li>• Algae, e.g. <i>Enteromorpha</i> or <i>Ulva</i> spp.</li> <li>• Cnidaria, e.g., <i>Obelia</i> or <i>Pdocoryne</i> spp.</li> <li>• Bryozoan, e.g., <i>Bugula</i> spp.</li> <li>• Sponge</li> <li>• Scuds (<i>Gammarus</i> spp.)</li> </ul>	<ol style="list-style-type: none"> <li>7. Showing where sessile organisms inhabit on the horseshoe crab and their relative size</li> </ol>

<p><b>Anatomy</b></p> <ul style="list-style-type: none"> <li>• Gross/external anatomy include:             <ul style="list-style-type: none"> <li>○ Segments of the body                 <ul style="list-style-type: none"> <li>▪ Prosoma, opisthosoma, telson</li> </ul> </li> <li>○ Promsomal appendages:                 <ul style="list-style-type: none"> <li>▪ Chlicerae</li> <li>▪ Pedipalps</li> <li>▪ Walking legs</li> <li>▪ Swimmer legs</li> </ul> </li> <li>○ Opisthosomal appendages:                 <ul style="list-style-type: none"> <li>▪ Genital operculum</li> <li>▪ Gill operculum and book gills</li> </ul> </li> <li>○ Mouth &amp; gnathobase</li> <li>○ Anus</li> <li>○ Sensory organ</li> <li>○ Vision:                 <ul style="list-style-type: none"> <li>▪ lateral compound eyes</li> <li>▪ ocelli &amp; median eyes</li> </ul> </li> </ul> </li> <li>• Internal anatomy             <ul style="list-style-type: none"> <li>○ Circulation</li> <li>○ Digestive system</li> <li>○ Respiratory system</li> <li>○ Excretory system</li> <li>○ Nervous system</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>8. Dorsal image with labeled structures</li> <li>9. Ventral image with labeled structures</li> <li>10. Swimmer leg w/ specific segments</li> <li>11. Walking leg w/ specific segments (pedipalps included on sex comparison image)</li> <li>12. Detailed gill anatomy</li> <li>13. Cross section showing relations of main systems</li> </ul>
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<p><b>Sex comparison</b></p> <ul style="list-style-type: none"> <li>• Females: larger, mating scars, continuous molting</li> <li>• Males: eroded shell &amp; covered by epibionts due to no molts once adults, bulge claw on pedipalp leg used to hold female's opisthosoma</li> <li>• Difference in genital pores</li> </ul>	<p>14. Image showing differences in:</p> <ul style="list-style-type: none"> <li>• Pedipalps</li> <li>• Genital pores</li> <li>• Shell appearance</li> <li>• Egg cluster</li> </ul>
<p><b>Necropsy</b></p> <ul style="list-style-type: none"> <li>• How to begin dissection/What approach to use</li> <li>• Steps to view all important anatomy</li> <li>• Realistic description of:             <ul style="list-style-type: none"> <li>○ Circulation</li> <li>○ Digestive system</li> <li>○ Respiratory system</li> <li>○ Excretory system</li> <li>○ Nervous system</li> </ul> </li> </ul>	<p>15 - 28.) Illustrations of entire dissection with associated anatomy and systems</p>



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## VITA

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Katie Joy Bergdale was born on October 26, 1992 in Sioux Falls, South Dakota and raised in Sioux City, Iowa. She continued her education pursuing the major of Biological and Pre-Medical Illustration at Iowa State University. During her undergraduate, an appreciation formed for human anatomy, leading her to continue her career to become a medical illustrator. She graduated with honors and received a Bachelor's of Arts in 2015.

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